Marine forensics: A molecular tool for trade monitoring and compliance in southern African fisheries, with focus on commercially exploited elasmobranch species

by

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Declaration

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Date: December 2019

Summary

Marine forensic science can be described as protecting fisheries resources, marine mammals and endangered species based on enforcement of the nation's laws. Species identification becomes challenging when morphological features (such as fins, scales and heads) are removed, or if confiscated samples are already in a processed state. The harvesting of elasmobranchs (sharks, rays and skates) is driven by the international shark meat and shark fin trade. In recent decades, a combination of increasing demand and economic globalisation has created a global market for elasmobranch products, especially the highly prized shark fins for Asian markets. In this study, marine forensics was assessed as a tool for complementing traditional identification methods – through the development of a mini-barcoding assay as well as investigating High Resolution Melting (HRM) analysis – for species identification, with focus on elasmobranch species occurring in the southern African region. Firstly, this involved the testing and optimisation of the standard barcoding region of the cytochrome oxidase *c* subunit I (*COI*) gene, and then using traditional barcoding primers as well as nested polymerase chain reaction (PCR) primers in a multiplex assay. Preliminary results (only a 22 % species identification success rate) indicated the limitations of using only the traditional *COI* primers and warranted the inclusion of alternate *COI* gene fragments for species identification in future related forensic cases. A mini-barcoding multiplex assay, comprising three primer sets, was optimised and applied to a wide range of forensic case studies involving confiscated shark fins, possibly for illegal trade. A significant number of CITES-listed and endangered species were identified when confiscated specimens from various regions in southern Africa were tested. Secondly, PCR amplification of a 16S ribosomal RNA (*16S rRNA*) gene fragment was optimised based on six southern African houndshark species and seven other commercially exploited species, including: hammerhead sharks *Sphyrna lewini* and *Sphyrna zygaena*, copper shark *Carcharhinus brachyurus*, dusky shark *Carcharhinus obscurus*, bull shark *Carcharhinus leucas*, blacktip shark *Carcharhinus limbatus* and blue shark *Prionace glauca*. High Resolution Melting analysis using the *16S rRNA* gene region was investigated as a species identification method for these species. The HRM assay was successfully applied for the identification of seven commercially exploited shark species, including some of the top commercially important sharks and one endemic houndshark *Scylliogaleus quecketti*. Although further

optimisation is required, this relatively fast and cost-effective approach will be a valuable tool for the initial screening of detained shipments, for possible illicit trade. Accordingly, this research presents species identification assays suitable for various shark related forensic case studies, and in future could be applied to identify most, if not all, elasmobranch species involved in trade regionally.

Opsomming

Mariene forensiese wetenskap kan beskryf word as die beskerming van visserye, mariene soogdiere en bedreigde spesies gebaseer op die handhawing van die land se wette. Spesies identifikasie word ‘n uitdaging wanneer morfologiese kenmerke (vinne, skubbe en koppe) verwyder word of in die geval waar gekonfiskeerde monsters reeds in 'n verwerkte toestand is. Die oes van spesies in sub-klas elasmobranchii (haaie en rogspesies) word deur die internasionale haaivleis- en haaivin-handelmark aangedryf. In onlangse dekades het ‘n kombinasie van toenemende vraag en ekonomise globalisering ‘n globale mark vir elasmotak-produkte geskep, veral die hooggewaardeerde haaivinne vir Asiatiese markte. In hierdie studie was mariene forensiese wetenskap beoordeel as ‘n instrument om tradisionele identifikasiemetodes te ondersteun – deur die ontwikkeling van ‘n mini-vingerafrdukmerker toets asook ‘n ondersoek van Hoë Resolusie-Smelting (HRS) analise – vir die identifisering van spesies, met klem op elasmobranchii wat in die suider-Afrika streek voorkom. Eerstens was die standaard strepieskode-streek van die sitokroom oksidase *c* subeenheid I (*COI*) geen geanaliseer en optimiseer, daarna is tradisionele vingerafdrukmerker inleiers sowel as geneste polimerase kettingsreaksie (PKR) inleiers in 'n veelvoudige metode getoets. Beperkings in die gebruik van slegs die tradisionele *COI* inleiers was beklemtoon deur voorlopige resultate (22 % spesies identifikasie sukses) wat die insluiting van alternatiewe *COI* geenfragmente vir die identifisering van spesies in toekomstige forensiese verwante gevalle geregverdig. ‘n Mini-vingerafdrukmerker veelvoudige toets, bestaande uit drie inleier-stelle, was geoptimiseer en op ‘n wye reeks forensiese gevallestudies toegepas, insluitend die betrokkenheid van gekonfiskeerde haaivinne – moontlik betrokke vir onwettige handel. ‘n Beduidende aantal CITES-gelysde en bedreigde spesies was geïdentifiseer toe monsters, gekonfiskeer van verskeie streke in suider-Afrika, getoets was. Tweedens, PKR amplifisering van 'n 16S ribosomale RNA (*16S rRNA*) geen fragment was geoptimiseer op grond van ses Suider-Afrikaanse sloothaaispesies en sewe ander spesies wat kommersieel ontgin word. Die sewe spesies van belang sluit in: hamerkophaaie *Sphyrna lewini* en *Sphyrna zygaena*, bronshaai *Carcharhinus brachyurus*, skemerhaai *Carcharhinus obscurus*, bulhaai *Carcharhinus leucas*, swarttiphaai *Carcharhinus limbatus* en blouhaai *Prionace glauca*. Hoë Resolusie-Smelting analise gekoppel met die *16S rRNA* geenstreek was ondersoek as ’n moontlike spesiesidentifiseringsmetode vir die spesies van belang. Die HRS

analise was suksesvol toegepas op die identifisering van sewe haaispesies wat kommersieel ontgin is, met van die mees kommersiële belangrike haaie en een endemiese sloothaai *Scylliogaleus quecketti* ingesluit. Alhoewel verdere optimiseering benodig is, is hierdie relatiewe vinnige en koste-effektiewe benadering 'n waardevolle hulpmiddel vir die aanvanklike keuring van aangehoude skeepsvrag vir moontlike onwettige handel. Gevolglik bied diè navorsing spesiesidentifikasie analises aan wat geskik is vir verskeie haai-verwante forensiese gevallestudies, en kan in die toekoms toegepas word om die meeste, indien nie alle, soort elasmobranchii-spesies wat by streekhandel betrokke is te identifiseer.

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Preface

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1. *Published or submitted papers, to date, directly emanating from the work presented in this thesis:*

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2. *Published or submitted papers with indirect relevance to the work presented in this thesis:*

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3. *Local conference presentations:*

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4. *International conference presentations:*

Asbury TA*, da Silva C, Gledhill KS, Maduna SN, Bester-van der Merwe AE. Oral presentation: Marine forensics: A molecular tool for trade monitoring and compliance in southern African fisheries, focussing on commercially exploited elasmobranch species. *3rd Sharks International*. June 2018. João Pessoa, Brazil.

Asbury TA, Bester van der Merwe AE*. Oral presentation: Shark and Ray Forensics, a conservation tool for the Western Indian Ocean region. *23rd European Elasmobranch Association Meeting*. October 2019. University of Calabria, Rende, Italy.

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List of Abbreviations

%	Percentage
/	Or
<	Less than
=	Equal to
>	Greater than
~	Approximately
3′	Three prime
5′	Five prime
16S rRNA	16S ribosomal RNA
$	United States Dollar
®	Registered Trademark
A	Adenine
AD	Anno Domini
BOLD	Barcode of Life Data System
bp	Base pairs
°C	Degrees Celsius
C	Cytosine
C. bra	*Carcharhinus brachyurus*
C. leu	*Carcharhinus leucas*
C. lim	*Carcharhinus limbatus*
C. obs	*Carcharhinus obscurus*
CAF	Central Analytical Facility
CITES	The Convention on International Trade in Endangered Species
cm^2	Square centimetre(s)
COI	Cytochrome *c* oxidase subunit I

CTAB	Cetyltrimethylammonium bromide
cytb	Cytochrome *b*
DAFF	Department of Agriculture, Forestry and Fisheries
DEA	Department of Environmental Affairs
dF/dT	Derivative of fluorescence over temperature
DNA	Deoxyribonucleotide Acid
dNTPs	Deoxynucleotide Triphosphates
DoH	Department of Health
dsDNA	Double-stranded DNA
°E	Degrees East
eDNA	Environmental DNA
EEZ	Exclusive Economic Zone
e.g.	*Exempli gratia* (for example)
EtBr	Ethidium bromide
F	Fluorescence
F	Forward primer
FAO	United Nations Food and Agriculture Organisation
FISH-BOL	Fish Barcode of Life
G	Guanine
G. gal	*Galeorhinus galeus*
GCP	Genotype Confidence Percentage
gDNA	Genomic DNA
HRM	High Resolution Melting
ID	Identification
i.e.	*Id est* (in other words)
IgE	Immunoglobulin E
Inc.	Incorporation

IPOA-Sharks	International Plan of Action for the Conservation and Management of Sharks
ITS2	Internal transcribed spacer 2
IUCN	International Union for the Conservation of Nature
kg	Kilogram
km	Kilometre
Ltd.	Limited company
m	Metre
M. mos	*Mustelus mosis*
M. mus	*Mustelus mustelus*
M. pal	*Mustelus palumbes*
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium chloride
min	Minutes
MLRA	Marine Living Resources Act
mM	Millimole
MPA	Marine Protected Area
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ND2	Nicotinamide adenine dehydrogenase subunit 2
ND4	Nicotinamide adenine dehydrogenase subunit 4
ng	Nanogram
ng/µl	Nanogram per microlitre
nm	Nanometre
No.	Number
NP	National Park
NPOA-Sharks	National Plan of Action for Sharks
NRF	National Research Foundation

P. gla	*Prionace glauca*
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
R	Reverse primer
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RSA	Republic of South Africa
°S	Degrees South
s	Seconds
S. lew	*Sphyrna lewini*
S. que	*Scylliogaleus quecketti*
S. zyg	*Sphyrna zygaena*
SA NPOA-Sharks	South African National Plan of Action for Sharks
SANBI	South African National Biodiversity Institute
SE	South East
Spp.	Species
SU	Stellenbosch University
SW	South West
T	Temperature
T	Thymine
T_A	Annealing temperature
TAC	Total Allowable Catch
TAE	Total Allowable Effort
Taq	*Thermus aquaticus* DNA polymerase
Tm	Melting temperature
TOPS	Threatened or Protected Species
U	Units (enzyme)

US	United States
V	Volts
v.	Version
WHO	World Health Organisation
WPC	World Parks Congress
vs.	Versus
w/v	Weight per volume
µl	Microlitre
µM	Micromole
X	Times (solution)

CHAPTER 1

Introduction: Literature survey, research aims and objectives

1.1 Elasmobranchs: Regional biodiversity, marine environment and drivers of threat

Cartilaginous fish belong to the class Chondrichthyes, a highly diverse class comprising two subclasses: Holocephali (chimaeras) and Elasmobranchii (sharks, skates and rays) (Carroll 1988; Maisey 2012; Ebert and van Hees 2015). All modern sharks and rays belong to the subclass Elasmobranchii, with fossil evidence suggesting that sharks date back to approximately 420 million years ago, making them one of the oldest surviving vertebrate lineages (Compagno 1990; Maisey 2012; Dulvy *et al*. 2017). Elasmobranchs radiated and became widely distributed in the world's oceans during the Age of Fishes (Early Devonian period), evolving to exhibit great diversity mechanically and morphologically (Miller *et al*. 2003; White and Last 2012; Dulvy *et al*. 2017), and inhabiting pelagic, demersal and coastal habitats (Compagno 1990; Heupel *et al*. 2014). Sharks have survived five mass extinction events, near the end of the Ordovician, Devonian, Permian, Triassic and Cretaceous Periods (Raup and Sepkoski 1982; Jablonski 1994), thus making them one of the most evolutionarily successful lineages historically. Over the last decade, approximately 230 new chondrichthyan species have been described. The majority of these newly described chondrichthyan species are reported from the western North Pacific, Indo-Australian and southern African regions (Ebert and van Hees 2015). The most recent data available indicates that known elasmobranch species worldwide consists of 517 species of sharks (nine orders, 34 families and 105 genera) and 646 species of batoids including rays and skates (four orders, 27 families and 104 genera) (Weigmann 2017).

Many studies suggest that sharks, as apex predators, are keystone species that have a significant influence within their marine ecosystems through top-down effects (Stevens *et al*. 2000; Myers *et al*. 2007; Ferretti *et al*. 2010). Globally, many shark species are experiencing severe population declines due to anthropogenic effects, such as pollution and overfishing, which lead to the degradation and overexploitation of coastal and marine habitats (Dulvy *et al*. 2014; Davidson *et al*. 2016). A decline of

one or more components within a food web could result in changes in abundance and food web connectivity with other species (Heithaus *et al*. 2008; Baum and Worm 2009; Dunne and Myers 2009). More specifically, a reduction of a predator population can influence the trophic network directly (abundance of prey) and indirectly (exclusion competition, apparent competition, behaviour effects), causing a trophic cascade. For example, if populations of intermediate components or "mesopredators" within the food web increase, it may consequently cause a reduction of lower trophic level populations or mesopredators' prey (Terborgh and Winter 1980; Soulé *et al*. 1988; Heithaus *et al*. 2008), thus, altering an essential balance among species and the overall biodiversity of oceans (Dudgeon *et al*. 2012).

The oceanographic regime of southern Africa is dominated by two major ocean current systems: the warm Agulhas Current along the Indian Ocean (east coast) and the cold Benguela Current along the Atlantic Ocean (west coast) (Griffiths *et al*. 2010). These regimes overlap between Cape Point and Cape Agulhas, creating a transition zone, or the Atlantic/Indian Ocean boundary (Griffiths *et al*. 2010; Teske *et al*. 2011). The diverse 3 650 km southern African coastline comprises sandy beaches, rocky shores, estuaries, mangroves, kelp forests, coral reefs and ocean depths of more than 5 km (Lombard *et al*. 2004; Griffiths *et al*. 2010; Teske *et al*. 2011). The South African coastline has been divided into nine marine bioregions, shown in **Figure 1.1**. Of these, five inshore bioregions are defined by faunistic and floristic classification, and four offshore bioregions are defined by physical criteria (e.g. depth and temperature; **Figure 1.1**) (Lombard *et al*. 2004).

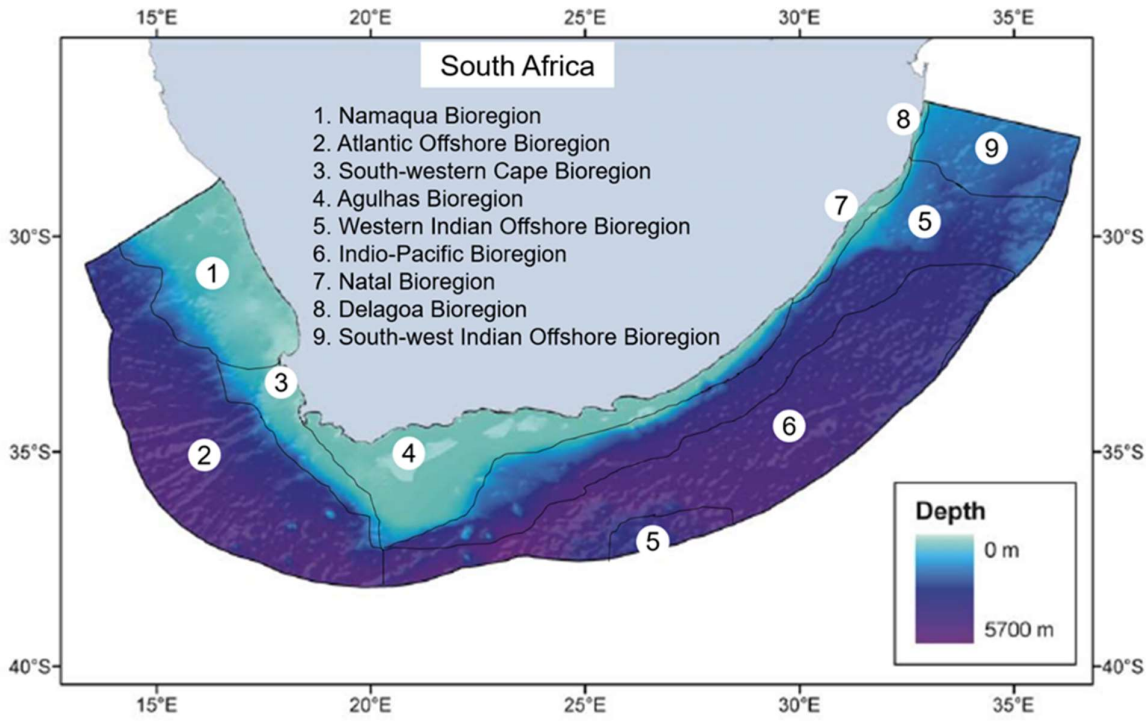


Figure 1.1: Map illustrating South Africa's nine marine bioregions (five inshore and four offshore bioregions) and seafloor depths, as described by Lombard (2004). Figure adapted from Griffiths *et al*. (2010).

The number of species currently described in the South African coastal marine bioregion is ~ 13 000 (Griffiths *et al*. 2010; Branch *et al*. 2016). Approximately 6 % of coastal marine species known globally occur in southern Africa waters, with over 35 % of these species being endemic to the region (Branch *et al*. 2016). Additionally, southern African marine habitats comprise 20 % of known chondrichthyans, with Southeast Africa representing one of the main shark global hotspots of high species richness, functional diversity and high endemism (Lucifora *et al*. 2011; Ebert and van Hees 2015). Of the 13 000 marine species present, approximately 204 are chondrichthyan species (117 shark, 79 batoid and eight chimaera species). Thus, southern Africa has one of the most diverse chondrichthyan faunas in the world, with at least 13 % of these species being endemic to the region (Ebert and van Hees 2015).

Worldwide, threats to marine biodiversity are driven by the rapid expansion of fisheries and global trade markets, whereby overfishing activities and habitat degradation have severely altered shark and ray populations (Stevens *et al*. 2000; Dudley and Simpfendorfer 2006; Ferretti *et al*. 2010). The main threat to chondrichthyans is overexploitation due to incidental catch and target fisheries, followed

by habitat loss and climate change (Dulvy *et al*. 2014). Chondrichthyans caught incidentally are often retained as they are frequently valuable bycatch of fisheries (Stevens *et al*. 2005). More recently, the harvesting of elasmobranchs is driven by the increasing value of fins, meat and livers (Fowler *et al*. 2002; Clarke *et al*. 2006b; Lack and Sant 2009). This has resulted in one third of threatened elasmobranchs being subjected to targeted fishing (Dulvy *et al*. 2014). In particular, shark fins are part of a globalised trade to meet the demand for shark fin soup, a traditional and expensive Chinese dish in Asian countries (Clarke *et al*. 2006a). The extreme life histories of chondrichthyans such as late maturity, slow reproduction, long gestation, low fecundity and high maternal investment, makes these species vulnerable to overexploitation (Cortés 2000; Frisk *et al*. 2001; Ferretti *et al*. 2010), thus limiting their ability to recover from ongoing threats.

In South Africa, chondrichthyans are targeted or caught as bycatch by eight out of sixteen commercial fisheries (da Silva *et al*. 2015). Approximately 50 % of chondrichthyan species that occur in southern Africa were reported in fisheries landings between 2010 and 2012, excluding chondrichthyan catches discarded at sea (da Silva *et al*. 2015). Although shark-processing factories observe realistic catch numbers, only an estimated 25–50 % of total shark landings are reported by fisheries (da Silva 2007). According to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, one quarter of chondrichthyan species are listed as threatened (Dulvy *et al*. 2014). Consequently, due to the threats chondrichthyans are facing in the southern African region, this coastline has been identified as a conservation priority (Dulvy *et al*. 2014; Davidson and Dulvy 2017; Stein *et al*. 2018), thus highlighting the importance of assessing the harvesting of Chondrichthyes in southern African waters.

1.2 Protection of elasmobranchs in South Africa

The protection for international trade of threatened shark species exists under The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). CITES is the first line of defence against illegal wildlife trafficking, listing over 35 000 plant and animal species with 183 involved parties (i.e. signatory nations). This is a binding and multilateral environmental agreement

between governments, with the aim to ensure that the survival of endangered wild plants and animals is not threatened by international trade. Species covered by CITES are listed in two major appendices according to their conservation status. Appendix I listing has the objective of prohibiting trade of wildlife species in immediate danger of extinction and Appendix II has the objective of preventing additional threatened species reaching a critical stage, by regulating trade so that it is legal, traceable and sustainable (CITES 2012). Approximately 96 % of all CITES-listed species are included in Appendix II and exporting parties have the obligation to document throughout the supply chain that traded specimens were legally obtained, and that trade is not detrimental to the survival of the species (CITES 2017). Globally, approximately 50 chondrichthyan species are reported in CITES lists (CITES 2012; CITES 2017; CITES 2019). These include seven sawfish species (*Pristidae* spp.) in Appendix I since 2007; and all manta (two *Manta* spp.) and devil rays (eleven *Mobula* spp.) are CITES-listed in Appendix II, since 2014 and 2017 respectively. There are fourteen CITES-listed shark species in Appendix II. Between 2001 and 2004, the first round of shark listings included three characteristically large-bodied and iconic species: the basking shark *Cetorhinus maximus*, whale shark *Rhincodon typus* and great white shark *Carcharodon carcharias*. Products of these species have not been identified in recent studies of major shark fin and meat markets (Jabado *et al*. 2014; Fields *et al*. 2018), suggesting they are rare in trade. The second round of shark listings included the porbeagle *Lamna nasus*, oceanic whitetip *Carcharhinus longimanus*, scalloped hammerhead *Sphyrna lewini*, great hammerhead *Sphyrna mokarran*, smooth hammerhead *Sphyrna zygaena*, bigeye thresher *Alopias superciliosus*, pelagic thresher *Alopias pelagicus*, common thresher *Alopias vulpinus* and silky shark *Carcharhinus falciformis* (from 2013–2016). This second group of species are all smaller at maturity than basking, whale and great white sharks, and studies have shown them to be more common in trade, both before and after being listed by CITES (Cardeñosa *et al*. 2018a; Fields *et al*. 2018). Recently, in 2019, the sharks shortfin mako *Isurus oxyrinchus* and the longfin mako *Isurus paucus* have been listed by CITES in Appendix II. Additionally, six giant guitarfish (*Glaucostegus* spp.) and ten wedgefish species (*Rhinidae* spp.) are now CITES-listed in Appendix II.

In South Africa, in addition to complying to CITES, the Department of Environmental Affairs (DEA; South Africa, Government department) developed a list of Threatened or Protected Species (TOPS). Originally, species that were critically endangered and animals facing an extremely high risk of extinction in the near future were listed, and reviewed every five years (TOPS 2015). To date, there are fourteen elasmobranch species on the TOPS list. The TOPS list has species categorically listed, based on IUCN Red List of Threatened Species classifications. Firstly, the elasmobranchs classified as Critically Endangered are: all species within the Family *Pristidae* (sawfishes), the Natal shyshark *Haploblepharus kistnasamyi* and whitespotted wedgefish *Rhynchobatus djiddensis*. Secondly, the Endangered species listed are: scalloped hammerhead *Sphyrna lewini* and great hammerhead *Sphyrna mokarran*. Thirdly, the Vulnerable species listed are: ragged-tooth shark *Carcharias taurus*, great white shark *Carcharadon carcharias*, whale shark *Rhincodon typus*, basking shark *Cetorhinus maximus* and flapnose houndshark *Scylliogaleus quecketti*. Lastly, the Protected species listed are: tiger shark *Galeocerdo cuvier*, leopard catshark *Poroderma pantherinum*, striped catshark *Poroderma africanum* and sixgill sawshark *Pliotrema warren* (DEA 2017). For all above-mentioned TOPS-listed species the following activities are restricted: hunting or killing and growing or breeding. Additionally, the following is restricted: capturing, having in possession/sanctuary/rehabilitation facilities, conveying/translocating, feeding, harassment and release – all except for scientific, management, conservation or rehabilitation purposes (DEA 2017).

In South Africa, all fisheries and the processing, sale and trade of marine resources are regulated by the Department of Agriculture, Forestry and Fisheries (DAFF; South Africa, Government department), under the Marine Living Resources Act (MLRA) (Act No. 18 of 1998, RSA 1998). South Africa follows guidelines from the International Plan of Action for the Conservation and Management of Sharks (IPOA-Sharks) (FAO 1999) as well as guidelines set up by the Food and Agriculture Organisation (FAO) Code of Conduct for Responsible Fisheries (FAO 1995). The IPOA-Sharks requires that a National Plan of Action for Sharks (NPOA-Sharks) is composed. In November 2013, the South African National Plan of Action for Sharks (SA NPOA-Sharks) was finalised with the goal to

move towards effective management and conservation of sharks occurring in the South African Exclusive Economic Zone (EEZ). It ultimately aims at improving optimal, sustainable and long-term use of sharks for the benefit of all South Africans, including present and future generations. More specifically, SA NPOA-Sharks ensures that: shark catches from all fisheries are sustainable; threats to shark populations are assessed with special attention given to vulnerable or threatened shark stocks; incidental catches of sharks, waste and discards from shark catches are minimalised; the full use of dead sharks is encouraged; and lastly, that species-specific catch, landings and trade data is improved (DAFF 2013). A progress assessment of the SA NPOA-Sharks reported that the most progress was for *optimum use* (100 % completed) and *classification and assessment of species* (84 % completed). The least progress made was for *regulatory tools* (0 % completed), mainly due to a lack of assessments, capacity management and funding from the government (da Silva *et al*. 2018). Currently, based on the progress highlighted, the SA NPOA-Sharks is in the process of being updated with intended completion by the end of 2019 (da Silva *et al*. 2018).

In terms of the MLRA in South Africa, sharks may not be landed, transported, or disposed of with their fins removed, nor without a permit. For the following fisheries: recreational line, gillnet, beach-seine, demersal shark longline and pelagic longline, there are limits to catches and species-specific permits are required (da Silva *et al*. 2015). The Fisheries Management sector regulates entries into commercial fisheries and is limited by an allocation process whereby scientific recommendations are taken into account. The number of vessels, crew and Total Allowable Catch (TAC) or Total Allowable Effort (TAE) is limited for target species, as well as catch limits for bycatch species (DAFF 2013). Additionally, for marine species protection, the South African coastline has regions acknowledged as Marine Protected Areas (MPAs), aiming to provide sanctuaries for highly resident fish, including shark species, and conserve biodiversity hotspots (DAFF 2013). International recommendations by the IUCN World Parks Congress suggests that at least 20–30 % of each habitat includes strictly protected areas (WPC 2003). For South Africa, in 2004, 23 % of the country's coastline fell within MPAs. However, only 9 % of the coastline's protected areas were fully protected

(or no-take MPAs). In offshore regions, less than 1 % fell within the country's EEZ ocean range and of this, less than 0.2 % was no-take. Additionally, the MPAs were not evenly distributed among South Africa's marine bioregions, for example, no MPAs were demarcated to the Namaqua bioregion on the west coast (Lombard *et al*. 2004). Since then, an additional twenty MPAs were proposed and officially declared in May 2019, including Namaqua bioregions (DEA 2019). Currently, 41 MPAs exist along the South African coast (South Africa EEZ) and are indicated in **Figure 1.2**.

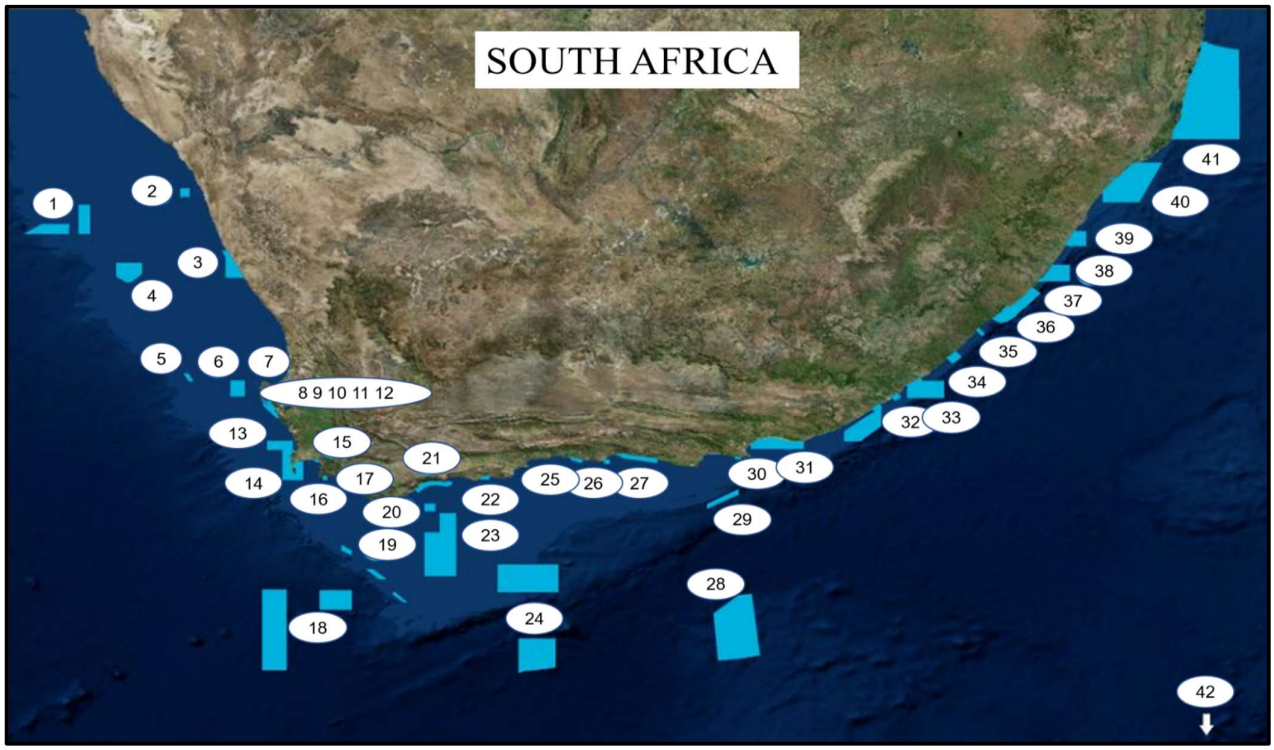


Figure 1.2: Marine Protected Areas (MPAs) along the South African coastline, adapted from: www.marineprotectedareas.org.za (South African National Biodiversity Institute – SANBI).

1-Orange Shelf Edge **2**-Namaqua Fossil Forest **3**-Namaqua National Park (NP) **4**-Childs Bank **5**-Benguela Mud **6**-Cape Canyon **7**-Rocherpan **8**-Malgas Island **9**-Marcus Island **10**-Jutten Island **11**-Langebaan Lagoon **12**-Sixteen-Mile Beach **13**-Robben Island **14**-Table Mountain NP **15**-Helderberg **16**-Betty's Bay **17**-Walker Bay **18**-SE Atlantic Seamounts **19**-Browns Bank Corals **20**-Agulhas Mud **21**-De Hoop **22**-Stilbaai **23**-Agulhas Bank Complex **24**-SW Indian Seamounts **25**-Goukamma **26**-Robberg **27**-Tsitsikamma **28**-Agulhas Front **29**-Port Elizabeth Corals **30**-Sardinia Bay **31**-Addo Elephant NP **32**-Amathole **33**-Amathole Offshore **34**-Dwesa-Cwebe **35**-Hluleka **36**-Pondoland **37**-Trafalgar **38**-Protea Banks **39**-Aliwal Shoal **40**-Uthukela Banks **41**-Isimangaliso **42**-Prince Edward Islands.

This recent increase in MPAs has dramatically increased South Africa's EEZ marine ecosystem area under protection from 0.4 % to 5.4 %. This contributes significantly to South Africa's international

commitments to marine ecosystem protection as well as providing protection to 90 % of marine habitat types (DEA 2019). The success of MPAs for shark species are observed for the Langebaan Lagoon MPA (**Figure 1.2**; region 11) and the De Hoop MPA (**Figure 1.2**; region 21), where coastal shark species such as ragged-tooth shark, cow shark, catsharks, smoothhounds and juvenile requiem sharks are offered partial protection (DAFF 2013). However, MPAs require adequate enforcement in order to conserve the species and habitats within their boundaries which has been a concern in South Africa (Griffiths *et al*. 2010). The effectiveness of MPAs as a tool for shark and ray conservation was assessed worldwide, it was concluded that MPAs are generally viewed as insufficient in isolation and social factors (local support, enforcement and management) are recognised as paramount for MPA success (MacKeracher *et al*. 2019).

In summary, South Africa complies to CITES and its own TOPS list which includes the protection of additional shark species, also comprising endemic sharks. Additionally, a large increase in MPAs across the country's EEZ has recently been approved. South Africa has well-developed fisheries and functional management structures, with prominent industrialisation. Even though complex fisheries practices may occasionally lead to lack of reliable and standardised management of chondrichthyan catch data, South Africa has relatively higher conservation scores and management compared to other international priority countries (Davidson and Dulvy 2017).

1.3 A global and local perspective of the shark finning industry

Shark finning is the act of removing and retaining shark fins, then discarding the remainder of the carcass at sea. Shark fin is commonly known as "yu chi" in China, and has been a delicacy in Chinese cuisine since the Song dynasty in AD (*Anno domini*) 960–1279 and established as the traditional dish "shark fin soup" in formal banquets, by the Ming dynasty in AD 1368–1644 (Rose 1996). From a global perspective, sharks have been caught and consumed for hundreds of years worldwide (Dent and Clarke 2015). However, in recent decades, a combination of increasing demand and economic globalisation has created a global market for shark products, especially the highly-valued shark fins for Asian markets. The meat of the same captured sharks is diverted to separate supply

channels, where the largest consumers of shark meat are found in Europe and South America – specifically Italy, Spain, Uruguay and Brazil (Dent and Clarke 2015) (**Figure 1.3**). The majority of shark fins are consumed in East Asia and Southeast Asia, particularly in China, Hong Kong, Taiwan, Vietnam and Malaysia (Dent and Clarke 2015) (**Figure 1.4**). From 2000–2011, Hong Kong maintained its position as the world's largest trader of shark fins with an estimated 10 480 tonnes of shark fins imported per year, worth an average US $378 million, and representing 80 % of the global total fin imports (Dent and Clarke 2015). Globally in 2011, the last year for which full global data is available, the average declared value of total shark fin imports was conservatively estimated by the FAO as US $438.6 million, for 17 154 tonnes imported (Dent and Clarke 2015). Subsequently, many countries prohibit or control shark finning including: The United States of America, Australia, Costa Rica, Brazil, the European Union, Oman and South Africa (Fowler and Séret 2010).

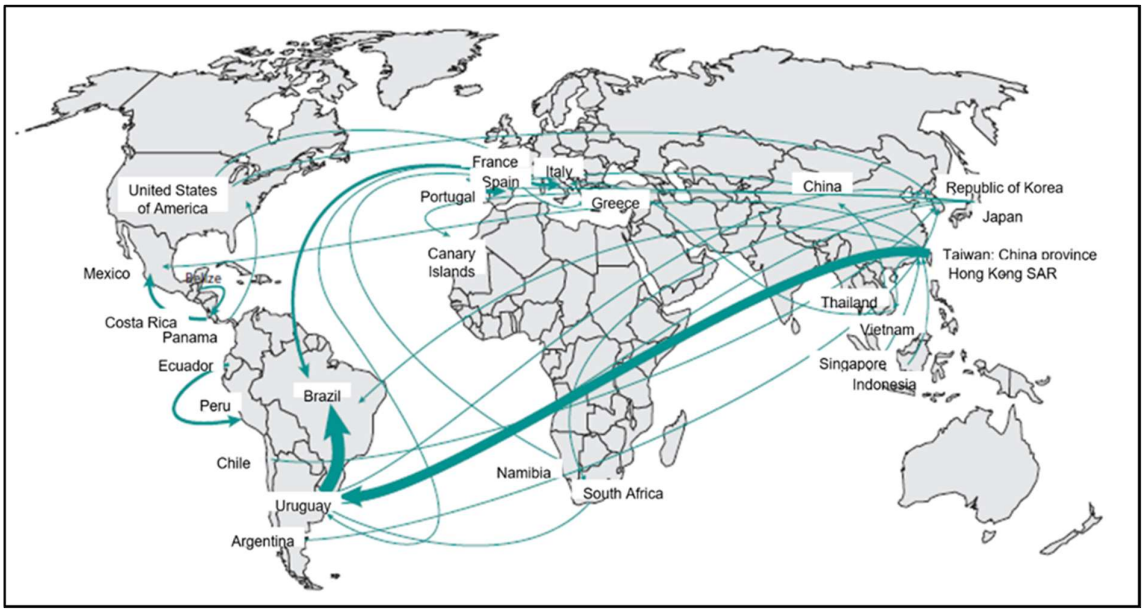


Figure 1.3: Map of global non-fin shark commodities trade, with all flows showing greater than 1 000 tonnes per year. Estimates based on FAO statistics of global trade flows of shark fins and other shark products from 2008–2011. Adapted from Dent and Clarke (2015).

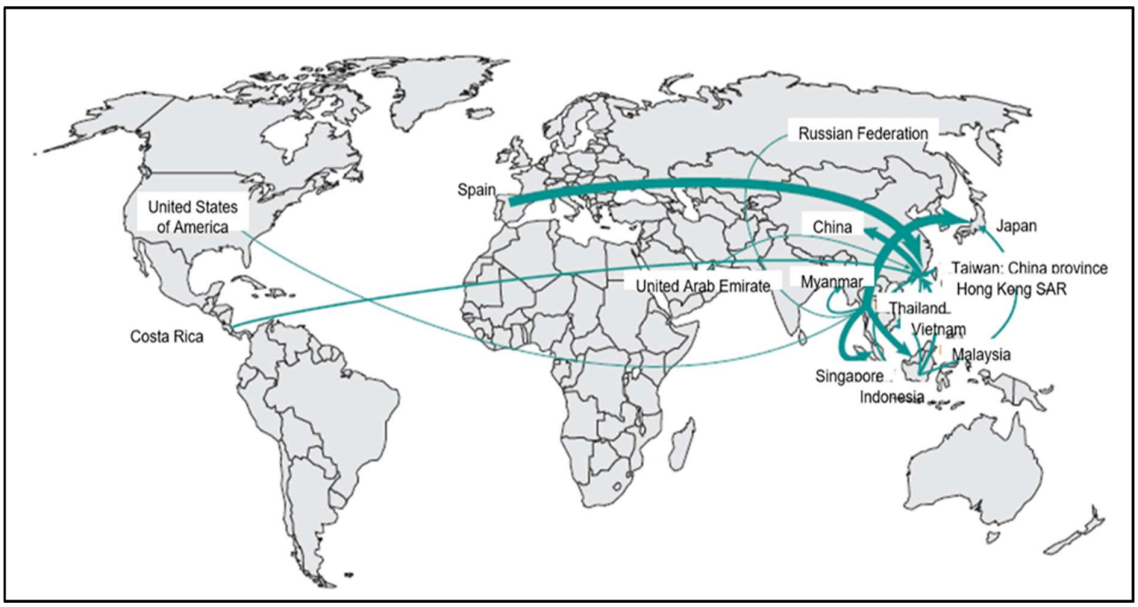


Figure 1.4: Map of global shark fin trade, with major flows showing greater than 300 tonnes per year. Estimates based on FAO statistics of global trade flows of shark fins and other shark products from 2008–2011. Adapted from Dent and Clarke (2015).

In South Africa, the exploitation of chondrichthyans on a commercial scale was initiated in the 1930s (von Bonde 1934), whereby an increased demand for natural vitamin A from shark liver was observed after the Second World War (van Zyl 1993). From 1945, annual shark landings exceeded 4 000 tonnes (van Zyl 1993). The industrial synthesis of vitamin A in 1967 caused a decrease in demand of shark products. The most heavily fished species, soupfin/tope shark *Galeorhinus galeus*, saw declines in numbers from the late 1940s (Davies 1964; Kroese and Sauer 1998). Subsequently, catches of *G. galeus* have not returned to pre-war levels (McCord 2005). A shark-directed longline fishery was established by 1992, whereby demersal and pelagic sharks were targeted (Kroese and Sauer 1998). Since then, annual shark landings have altered dramatically due to this fishery, along with price and demand (da Silva and Bürgener 2007). The legality regarding shark finning in South Africa is that current permit conditions prohibit the act of shark finning; however, fins may be removed provided that trunks are retained. An exception for two shark species exists, the shortfin mako *Isurus oxyrinchus* and blue shark *Prionace glauca*, whereby total weight of retained fins may not exceed 8 % and 13 % of the total weight of the trunks respectively (DAFF 2014). During the past two decades, the demand for South

African shark products (frozen, fresh or finned) has increased (da Silva and Bürgener 2007) due to the global increase in fin price (Clarke *et al*. 2006a). This incentive has caused the targeting of large sharks, regardless of their fillet value (da Silva *et al*. 2015). Irrespective of size, pelagic sharks (such as shortfin mako *I. oxyrinchus*) are valuable in both the fillet and fin trade. Whereas other shark species such as blue shark *P. glauca* and broadnose sevengill *Notorynchus cepedianus* are considered low-value due to strongly flavoured meat and absence of muscle firmness; however, they are targeted for their fins and livers (da Silva and Bürgener 2007). Demersal sharks are targeted for fillets and fins, whereby smaller sharks (2–7 kg) are generally of higher value due to lower mercury and cadmium levels. Larger demersal sharks are caught for fins and livers (da Silva and Bürgener 2007; DAFF 2012). Between 2010 and 2012, reported catch of chondrichthyans in South Africa averaged 3 000 tonnes with two-thirds landed as bycatch (da Silva *et al*. 2015). The most recent report is for 2016, where catch of chondrichthyans in South Africa was 2 300 tonnes (da Silva *et al*. 2018), indicating a decrease in catch.

In terms of FAO global economic statistics driven by shark-derived products, the imports of shark fins from 2000–2004 for South Africa, was an average of 13 tonnes per year (US $72 000), with no reported statistics available for 2004–2011. South Africa was ranked 15th in world for imports of shark fins (Dent and Clarke 2015). South Africa's export of shark meat from 2000–2011 was an average of 942 tonnes per year, and ranked 20th in the world for exports of shark meat (Dent and Clarke 2015). Additionally, South Africa is a major market for Japanese shark meat exports based on volume. From 2000–2012, exports of shark meat to South Africa increased more than 13-fold, from 124 tonnes (US $76 000) to 1 433 tonnes (US $638 000) (Dent and Clarke 2015). Shark fins from South Africa are exported to ten countries; with Hong Kong, Japan, Democratic Republic of Congo and Australia accounting for 98 % of fin exports (da Silva *et al*. 2015). Illegal finning activity in South Africa is confirmed by discrepancies between fin export data and import data to Hong Kong (da Silva *et al*. 2015). Furthermore, there is supporting evidence that large quantities of dried shark fins are exported illegally with the involvement of Chinese triad gangs, from Cape Town to Hong Kong and Southeast

Asia, *via* Johannesburg (Gastrow 2001). Thus, South African shark trade data could be a poor indicator of landed shark catches in the South African region.

Although shark finning is banned in South Africa and the country is not a major contributor to worldwide shark fin and meat trade, there are significant discrepancies for South Africa's shark trade data. Thus, shark catch statistics for South Africa are not always accurate; however, the country's current shark landings are well below the precautionary upper catch limit (da Silva *et al*. 2015).

1.4 Molecular tools used for species identification of elasmobranchs

Often, the first or only problem in fisheries enforcement is identifying the species to which a fish sample or product belongs (Ogden 2008). Visual species identification techniques exist; however, defining morphological features are removed during shark processing at sea (Abercrombie *et al*. 2005), thus making species identification difficult. Firstly, other morphological identification methods such as teeth and dermal denticle morphology have been used for a range of shark species (Valenzuela *et al*. 2008; Crooks *et al*. 2013; Maduna 2017) as well as guides such as an identification key for identifying gutted and headed sharks, with an existing guide for demersal sharks in South Africa (da Silva 2007). Secondly, for shark fin identification, automated visual identification from dorsal fin imagery of individual great white sharks (*C. carcharias*) has been developed (Hughes and Burghardt 2017). Additionally, a Shark Fin Guide covering sixteen globally distributed shark species was published, including CITES-listed species (such as for whale shark *R. typus* and great white shark *C. carcharias*) as well as shark species directly targeted for their fins (such as hammerhead sharks *Sphyrna* spp. and sandbar shark *Carcharhinus plumbeus*) (FAO 2016). However, these morphological and visual species identification techniques are labour intensive, require expert taxonomists and are not always possible as large numbers of specimens from fisheries are often landed. Many molecular techniques for genetic identification of body parts and multiplex polymerase chain reaction (PCR) methods have been established to identify shark specimens and products.

Every species has a unique DNA sequence that distinguishes it from all other species, the "DNA barcode", and a number of molecular fingerprinting techniques have been developed based on this fact offering the potential for the regulation and monitoring of fisheries (Ogden 2008). Genetic systems for molecular identification generally fall into four classes: the first class employs mitochondrial-based DNA sequences, the second class pertains to nuclear genetic markers, the third class makes use of microsatellite markers and the final class exploits environmental DNA (eDNA). The primary use of these techniques is molecular species identification through "DNA barcoding". As a bio-identification system for animals, a single gene sequence, the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene is used to differentiate majority of animal species (Hebert *et al*. 2003). Other gene regions such as cytochrome *b* (*cytb*) mitochondrial gene region as well as nicotinamide adenine dehydrogenase subunit 2 (*ND2*) and internal transcribed spacer 2 (*ITS2*) nuclear gene regions are also commonly targeted for species identification in fish (Pank *et al*. 2001; Shivji *et al*. 2002; Ogden 2008; Krück *et al*. 2013). Importantly, the *COI* barcoding region has been shown to discriminate the vast majority of fish species (Ward *et al*. 2005) and has been a powerful tool not only for fish species identification (Keskin and Atar 2013; Landi *et al*. 2014), but also to successfully identify shark species (Holmes *et al*. 2009; Fields *et al*. 2015; Hobbs *et al*. 2019). The identification of shark species using the *COI* gene region in a multiplex PCR as well as PCR-restriction fragment length polymorphism (PCR-RFLP) techniques have been employed (Mendonça *et al*. 2009; Pinhal *et al*. 2009). For the successful application of DNA barcoding, existing reliable reference libraries are required. To obtain species identity, sequences produced for specimen samples can be compared to reference sequences available on publicly available databases. Examples of these databases are GenBank (Benson *et al*. 2013) or the Barcode of Life Data System (BOLD; Ratnasingham and Hebert 2007), often resulting in species being identified by a tight cluster of sequences or a single sequence (Ward *et al*. 2008). The Fish Barcode of Life (FISH-BOL) campaign has the primary goal of collecting DNA barcodes for the world's fish species. Voucher specimens identified morphologically by taxonomic experts are used to obtain standard reference DNA sequences (Ward *et al*. 2005; Ekrem *et al*. 2007). These sequences can be used to broadly identify and

better characterise fish species; and reliable reference sequences generated from voucher specimens are especially important for correct species identification.

Additionally, for fisheries species identification of elasmobranchs, microsatellite markers have been utilised due to their high mutation rate and being highly polymorphic (Tautz 1989; Weber and Wong 1990). Microsatellite markers are repeated sequences of usually two to five nucleotides, and are one of the most powerful molecular genetic tools with an array of applications, including the identification of species and individuals (Martin *et al*. 2002; Sekino and Hara 2007; Costa *et al*. 2012). They have been applied previously for species identification of sharks and rays occurring in southern Africa (Martin *et al*. 2002; Maduna *et al*. 2014). However, when a large number of specimens from fisheries require processing, the use of microsatellite markers for species identification is not the most cost- and time-effective approach.

A more recent and cost-effective approach includes using High Resolution Melting (HRM) analysis, originally developed for single nucleotide polymorphisms by Wittwer *et al*. (2003). High Resolution Melting analysis is a post-PCR analysis, used for identifying genetic variance in nucleic acid sequences (Sharma *et al*. 2013). An intercalating dye binds to the PCR product and the fragments are subjected to incremental increasing temperature, causing denaturation of the double-stranded DNA (dsDNA), resulting in a decrease in fluorescence (Applied Biosystems 2010; Kapa Biosystems 2013). The fluorescence is detected and plotted as a melt curve, where each curve varies with the size and GC/AT ratio of the generated amplicon sequence (Ririe *et al*. 1997), as seen in **Figure 1.5**. This assay has been used to discriminate whaler shark species distributed in Australia (Morgan *et al*. 2011) and as a field-based tool for the identification of possible illegally imported or exported CITES-listed shark specimens (Cardeñosa *et al*. 2018b). High Resolution Melting analysis is a higher-throughput approach which is more suitable when a large number of samples need to be processed, it is a closed-tube assay and does not require post-PCR manipulations, making it more cost-effective (Morgan *et al*. 2011; Rugman-Jones and Stouthamer 2016).

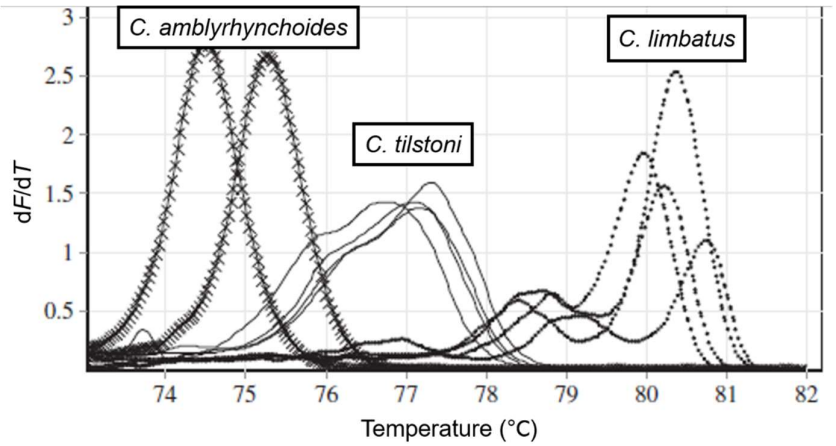


Figure 1.5: Melt curve profile examples for three whaler shark species (*Carcharhinus amblyrhynchoides*, *Carcharhinus tilstoni* and *Carcharhinus limbatus*), adapted from Morgan *et al*. (2011). Melt curves plotted as the negative derivative of fluorescence (F) over temperature (T) (dF/dT) vs. temperature (°C).

More recently, eDNA has proven to be a successful method for the detection and identification of present or elusive species in a wide range of ecosystems, including marine environments (Miya *et al*. 2015; Thomsen *et al*. 2016). DNA is extracted directly from an environmental sample (water, air, soil), without isolating a particular organism (Deiner *et al*. 2017; Taberlet *et al*. 2018), thereafter it is amplified, sequenced and assigned to its species of origin through metabarcoding (Ji *et al*. 2013). Analyses from eDNA can be a non-invasive approach to identify species or communities associated with that particular environment (Goldberg *et al*. 2015), and an alternative to track present species and abundance in their environment (Thomsen *et al*. 2012; Ji *et al*. 2013). Due to its limited persistence in water, eDNA extracted from water samples is relatively short lived and 100 bp eDNA fragments can degrade within days (Thomsen *et al*. 2012). Thus, making eDNA analysis a good candidate for biomonitoring of marine species, as it indicates recent presence in the environment (Jerde *et al*. 2011; Pilliod *et al*. 2014). Importantly, eDNA metabarcoding has been effectively employed to study shark diversity, whereby the first study to detect a single elasmobranch species using eDNA was for the detection of the endangered largetooth sawfish (*Pristis pristis*) (Simpfendorfer *et al*. 2016). Similarly, a species specific eDNA approach has also been applied to detect the whale shark (*R. typus*), and more recently the great white shark (*C. carcharias*), from oceanic water samples (Sigsgaard *et al*. 2016; Lafferty *et al*. 2018). The method of eDNA metabarcoding can also be employed to effectively identify

not only one species but many existing in a single environment simultaneously, and has been utilised to describe shark diversity across an ecosystem, as diverse as four locations in the Caribbean (Bakker *et al*. 2017). Disadvantages of eDNA includes that the non-detection of specific species does not imply the absence of those species, nor does a positive signal necessarily infer the presence of the species (Roussel *et al*. 2015). The use of eDNA cannot always detect contemporary species presence or show accurate representation of a community at a specific time (Collins *et al*. 2018). The concentration of DNA in the water samples may be beneath the threshold of detection due to dilution or degradation. Conversely, DNA detection could be due to the species passing through those waters or DNA transported by water movements (Roussel *et al*. 2015). Also, a recently developed portable sequencer or "genome skimming" device termed the "MinION", is able to obtain multiple barcode sequences for taxonomic identification as well as complete mitochondrial and nuclear genomes of chondrichthyans (Johri *et al*. 2019). The MinION device can be used in the field whereby the species identification of shark fin specimens from an Indian export market was performed (Johri *et al*. 2019). This method allows for the sequencing of larger regions (as opposed to single barcode sequencing) of chondrichthyan genomes, allowing for not only taxonomic identification but also population genetic and biogeographic studies, as well as stock assessments (Johri *et al*. 2019). Limitations of the MinION include high error rate, high levels of inter-run variability, it is relatively more expensive and there is frequent modification of the kits required (Tyler *et al*. 2018). Even though limitations exits, these more recent molecular approaches have the potential to monitor elusive marine predators, as well as allow for higher-throughput of specimens for species identification, ecosystem assessment and conservation management.

An alternative approach to the above-mentioned molecular methods for species identification is required when presented with samples containing degraded DNA from processing. While trained examiners and taxonomists can morphologically identify unprocessed fins, almost all defining morphological characteristics from fish species are removed during processing, making it impossible to identify them using traditional identification approaches (Ogden 2008). The processing of shark fins

initially entails removing the shark fin from the body carcass, followed by oven or sun drying the fins. Shark fins in the international trade to Asian markets are usually in this state (with the skin attached). Species identification based on morphological features is possible at this stage; however, standard DNA barcoding and PCR techniques are used to confirm species-of-origin when they cannot be morphologically identified (Chapman *et al*. 2003; Abercrombie *et al*. 2005; Wong *et al*. 2009; Fields *et al*. 2015). The next stage of processing first involves the removal of dermal denticles (outermost skin layer) and subsequently involves chemical treatment to produce the skinned and bleached "processed fin" product (Vannuccini 1999). During fin processing, the genomic DNA (gDNA) is degraded, thus making it challenging to obtain larger PCR amplicons (Fields *et al*. 2015). However, this problem can be solved by using multiple markers or multiple gene regions to produce more robust results, compared to a singular molecular marker (Feitosa *et al*. 2018), or by using a mini-barcoding assay (Fields *et al*. 2015; Shokralla *et al*. 2015; Cardeñosa *et al*. 2017). Obtaining short mini-barcodes of the *COI* barcoding region is a cost-effective method, when DNA sequence information from degraded DNA is required (Hajibabaei *et al*. 2006; Meusnier *et al*. 2008). A mini-barcoding approach has been proven successful for identifying processed shark products, such as whole processed fins and individual fin needles (ceratotrichia) extracted from shark fin soup, for most CITES-listed sharks (Fields *et al*. 2015; Cardeñosa *et al*. 2017).

Therefore, to conclude, when presented with a high number of samples from shark specimens a lower cost and higher-throughput method such as HRM analysis is ideal; whereas a mini-barcoding approach is more efficient when presented with processed shark products as the gDNA is often degraded, in turn, providing means for fisheries management and compliance.

1.5 Research aims and objectives

The overall aim of the research presented in this thesis is to develop molecular identification assays (mini-barcoding multiplex assay and High Resolution Melting assay) relevant to commercially exploited shark species occurring in southern Africa, in order to complement traditional methods used in fisheries management and compliance.

The first experimental chapter (Chapter 2) investigates shark species present in case studies involving confiscated illegal shark fins in southern Africa, and the correct labelling of fish products from retail outlets in the Western Cape, South Africa. Species identification is performed using a traditional method of barcoding as well as an optimised mini-barcoding multiplex assay. The study describes the findings from these case studies, which includes identifying a number of CITES-listed and endangered shark species from various regions in southern Africa. Furthermore, this allows for the detection of potential illicit trade in threatened species under the control of international protections (i.e. CITES-listed shark species), thereby contributing to the knowledge of internationally traded species.

In the second experimental chapter (Chapter 3), an HRM assay based on the 16S ribosomal RNA (*16S rRNA*) gene region is assessed for use in southern African commercially exploited shark species. This entails validation of the HRM assay for five houndshark species and further optimisation for another four commercially exploited shark species. It was concluded that the HRM assay can be applied as a functional species identification tool for several commercially important shark species including: *Scylliogaleus quecketti*, *Mustelus mustelus*, *Mustelus mosis*, *Carcharhinus limbatus*, *Prionace glauca*, *Carcharhinus brachyurus* and *Carcharhinus obscurus*. This assay requires further optimisation but could be used in conjunction with traditional specimen identification methods for shark forensic case studies consisting of a large number of samples (ideally of adequate quality). High Resolution Melting analysis is higher-throughput and more cost-effective compared to other species identification methods, therefore it has the potential to be used as an initial species identification measure.

CHAPTER 2

Mini-barcoding assay optimisation and application in forensic identification of southern African commercially exploited shark species

Abstract

DNA barcoding is a widely used method for identifying organisms based on a short, standardised fragment of genomic DNA – a region of the cytochrome *c* oxidase subunit I (*COI*) gene. It is a method that has proven to be effective in revealing the mislabelling of shark products, as well as identifying threatened and endangered shark species in the shark fin trade. In this study, a mini-barcoding assay relevant to elasmobranch species occurring in the southern African region was developed. This involved the testing and optimisation of the standard barcoding region of the *COI* gene, then using a traditional barcoding primer pair as well as two nested polymerase chain reaction (PCR) primers in a multiplex assay. Preliminary results based on the forensic analysis of unidentified confiscated shark fins illustrated the limitations of using only the traditional *COI* primers. With only a 22 % species identification success rate, it is possible that the genomic DNA was too degraded as is often the case with processed shark fins. This warrants the inclusion of alternate fragments of the *COI* gene region for species identification in future shark forensic cases. Accordingly, a mini-barcoding multiplex assay comprising five primers, amplifying three *COI* gene regions, was optimised and applied to a wide range of case studies. The case studies involved confiscated shark fins, from various locations in southern Africa, in which threatened, endangered as well as CITES-listed species were identified for possible illegal trade. Finally, the mini-barcoding assay was proven successful in identifying highly processed shark fin samples mostly to the species-level, but at least to the genus-level.

2.1 Introduction

Over the past few decades there has been overexploitation of sharks on a global-scale, primarily to supply international markets with products such as meat, skin, fins, cartilage, liver and teeth (Clarke *et al*. 2006a; Lack and Sant 2009; Dulvy *et al*. 2014). The most prominent of these is the 'shark fin trade' whereby the fins of sharks and shark-like rays (such as sawfishes and wedgefishes) are used for shark fin soup. This dish is a delicacy in some Asian countries and specifically in Hong Kong, which is considered a major fin trade hub (Fields *et al*. 2018). Worldwide there are fourteen major species targeted for the shark fin industry, namely: blue shark *Prionace glauca*, shortfin mako *Isurus oxyryhnchus*, silky shark *Carcharhinus falciformis*, dusky shark *Carcharhinus obscurus*, sandbar shark *Carcharhinus plumbeus*, tiger shark *Galeocerdo cuvier*, bull shark *Carcharhinus leucas,* scalloped, smooth and great hammerhead *Sphyrna lewini, S. zygaena, S. mokarran*, common, bigeye and pelagic thresher *Alopias vulpinus, A. superciliosus, A. pelagicus* and the oceanic whitetip shark *Carcharhinus longimanus* (Amaral *et al*. 2017). These species are targeted directly or caught as incidental bycatch (Worm *et al*. 2013; Oliver *et al*. 2015) and used to supply a market that is largely unmonitored and unregulated. Subsequently, more than half of the chondrichthyans that enter the fin trade are under threat (Dulvy *et al*. 2014). Sharks are vulnerable to overexploitation, not only due to overfishing but also owing to their *K*-selected life history characteristics such as slow growth, late maturity age, low fecundity and long gestation periods (Dapp *et al*. 2013; Dulvy *et al*. 2014; Hutchinson *et al*. 2015). Consequently, there is evidence for widespread shark and ray population declines (Davidson *et al*. 2016). First, a recent investigation of the sustainability of the reported global catch suggests that large predators, such as sharks, and other coastal species were already depleted by 1975 (Costello *et al*. 2012). Second, as a result from overfishing and according to the International Union for Conservation of Nature (IUCN), an estimated 25 % of all sharks and rays are threatened with increased extinction risk (Vulnerable, Endangered or Critically Endangered) (Dulvy *et al*. 2014). Even though a recent survey shows that at least 9 % of the current global catch of sharks are biologically sustainable (Simpfendorfer and Dulvy 2017), mounting evidence suggests that apex shark populations are more vulnerable to

exploitation than previously thought and ongoing declines are of a concern (Roff *et al*. 2018). The sustainability of sharks is especially important as they are the most evolutionary distinct fish lineages and play important functional roles in marine environments (Stevens *et al*. 2000). They have an important structural and functional role in the top-down control of coastal and oceanic ecosystems (Stevens *et al*. 2000; Heithaus *et al*. 2012). A decline in sharks could lead to trophic cascades through changes in prey abundance or behaviour, mesopredator release and declines in some commercial fish (Ferretti *et al*. 2010). Thus, the sustainability and monitoring of shark species is particularly important for stable and functional marine ecosystems.

For many commercially important shark species, catches are unregulated and species-level data is often poorly recorded, undermining the development for effective shark management strategies (Barker and Schluessel 2005). A lack of species-specific data (catch rate, annual landings and bycatch/discard level) stems from misidentified species or sharks that have been discarded at sea, as fisheries only report retained (landed) catch (Lack and Sant 2009; FAO 2014; Bonanomi *et al*. 2017). For multi-species fisheries, species identification during port inspections is highly challenging when using traditional morphological or taxonomic tools, as carcasses are often processed at sea, whereby morphological features such as heads and fins of specimens are removed (Abercrombie *et al*. 2005; Mendonça *et al*. 2010; Gulak *et al*. 2017). Additionally, morphological features are often similar between species – for example carcharhinid species such as *Carcharhinus limbatus* and *Carcharhinus tilstoni* (Tillett *et al*. 2012) – making discriminant species identification difficult. In the case of similar-looking elasmobranchs, species are often aggregated and data presented is of several species, thus making catch and landing data inaccurate (Dulvy *et al*. 2000; Barausse *et al*. 2014; Williams 2017). Consequently, aggregated data may conceal opposite trends of individual species, with the decrease of one species being compensated by an increase of another (Dulvy *et al*. 2000). Additionally, data from scientific surveys may also be confounded by misidentification when species lack an unambiguous phenotypic-based identification method (Marino *et al*. 2017).

In order to solve shark species identification issues, molecular-based methods have regularly been used over the last decade as alternatives to morphological identification (Amaral *et al*. 2017). These molecular techniques include protein and gel electrophoresis identification methods (Smith and Benson 2001; Farrell *et al*. 2009); DNA barcoding and sequence-based identification methods (Ward *et al*. 2005; Blanco *et al*. 2008); PCR multiplex methods (Farrell *et al*. 2009; Mendonça *et al*. 2010); and more recently, High Resolution Melting analysis (Morgan *et al*. 2011; Cardeñosa *et al*. 2018b). DNA barcoding makes use of universal primers targeting a short, standardised gene region (~650 bp for animal species) of the mitochondrial gene encoding cytochrome *c* oxidase subunit I (*COI*) (Hebert *et al*. 2003). Specifically, for elasmobranchs, the use of the *COI* gene region has proven to be successful for accurately identifying a broad range of species (Ward *et al*. 2008; Bineesh *et al*. 2017). Importantly, this method has also been effective in revealing the mislabelling of shark products, as well as identifying threatened and endangered species in the shark fin trade (Liu *et al*. 2013; Moore *et al*. 2014; Asis *et al*. 2016; Cardeñosa *et al*. 2017; Steinke *et al*. 2017). It is imperative that elasmobranchs are identified to the species-level, in order to account for population vulnerability and species susceptibility to exploitation, for effective fisheries management.

Furthermore, an issue of seafood fraud has previously been detected in South Africa whereby a study on fish fillets revealed that about half of all fillets tested were mislabelled (von der Heyden *et al*. 2009). An additional study showed 9 % of samples from wholesalers and 31 % from retailers were identified as different species to the ones indicated at the point of sale (Cawthorn *et al*. 2011). Other concerning factors were also highlighted based on the findings from these two studies, such as: species prohibited for sale in South Africa were being sold to consumers, substitution of fish species, as well as a 'product of South Africa' label present for an imported species (von der Heyden *et al*. 2009; Cawthorn *et al*. 2011). Thereafter, the seafood fraud investigation was revisited and found that the rate of seafood mislabelling for restaurants was 18 % and for retail outlets was 19 % (Cawthorn *et al*. 2015). Compared to the previous 2011 statistics, there seems to be an improvement in the transparency of local

seafood marketing. However, seafood fraud remains a concern and indicates the requirement for improved monitoring of seafood labelling and law enforcement in South Africa (Cawthorn *et al*. 2015).

In South African fisheries, the misidentification of shark species in fisheries operation is also a major concern (da Silva *et al*. 2015). Six demersal shark species commonly targeted in South Africa include: the common smoothhound *Mustelus mustelus*, whitespotted smoothhound *Mustelus palumbes*, tope shark *Galeorhinus galeus*, copper shark *Carcharhinus brachyurus*, dusky shark *Carcharhinus obscurus* and broadnose sevengill *Notorynchus cepedianus* (da Silva and Bürgener 2007; da Silva *et al*. 2015, 2018). When shark carcasses arrive at processing facilities, fins are removed, after which the sharks are filleted and skinned. The vast majority of processed demersal shark meat is exported to Australia primarily for the fish-and-chips trade, while the cartilage that is removed during filleting is sold to a buyer in South Africa or overseas for the complementary medicine sector (da Silva and Bürgener 2007). Shark fins are dried and exported to Hong Kong; however, this practice is not common but rather that facilities have trade contacts in Asia associated with other seafood (da Silva and Bürgener 2007). Shark fins can be found in numerous stages of processing. Firstly, wet fins are those that have been removed from a recently harvested shark (not dried or processed further) and still contain skin (Abercrombie *et al*. 2018). Secondly, most fins entering the international trade are dried but unprocessed and are those that are rigid, still containing both skin and cartilage. Both wet and dried, unprocessed fins generally contain genomic DNA (gDNA) of sufficient quality that can be amplified using PCR (Abercrombie *et al*. 2018). Lastly, fins can be processed, dried and chemically treated to remove the skin and are a yellow or golden colour. These processed fins often contain degraded gDNA, meaning DNA is broken down into very small DNA fragments, often incompatible with the use of standard genetic identification techniques (Abercrombie *et al*. 2018). In order to overcome this problem, a mini-DNA barcoding assay was recently developed by Cardeñosa *et al*. (2017), whereby shorter *COI* gene fragments are amplified in a nested PCR and used for species identification when dealing with processed shark fins.

Therefore, considering the heavy exploitation of sharks in South Africa and that shark fins are often in various states of processing; the aim of this chapter is to optimise a mini-barcoding multiplex assay, based on southern African commercially exploited shark species, and thereafter apply this assay and a traditional barcoding methodology to marine forensic case studies. The methods include (1) creating a positive control sample list of commercially exploited shark and ray species using a traditional barcoding method and (2) optimising a mini-barcoding multiplex assay (amplifying *COI* gene fragments ~150 bp, ~200 bp and ~650 bp) for southern African commercially exploited shark species. This chapter reports on the findings from marine forensic case studies involving the testing of fish products for mislabelling, from a variety of retail outlets, as well as species identification of confiscated shark fins from various regions in southern Africa. Ultimately, this allows for the detection of potential illicit trade in threatened species under the control of international protections (i.e. CITES-listed shark species); as well as contributing to the knowledge of internationally traded species.

2.2 Methodology

2.2.1 Positive control sample list

A positive control sample list comprising shark and ray species available across the research group (Molecular Breeding and Biodiversity Group, Genetics Department, Stellenbosch University) was established. DNA working stocks (50 ng/µl) were made from gDNA stocks if available, otherwise gDNA was extracted from fin clip samples stored in 99 % ethanol using an adjusted cetyltrimethylammonium bromide (CTAB) DNA extraction protocol (Sambrook and Russell 2001). DNA quantification (ng/µl) and quality (absorbance ratio: 260/280 and 260/230) was determined using a NanoDrop Spectrophotometer and concentrations adjusted accordingly to 50 ng/µl. The purity of DNA and RNA is assessed based on the ratio of absorbance at 260 nm and 280 nm. Generally, for DNA, a ratio of ~1.8 is acknowledged as "pure" and if the ratio is considerably lower it could indicate the presence of phenol, protein or other contaminants that absorb strongly at 280 nm. The 260/230 ratio is used as a secondary measure of nucleic acid purity and for "pure" nucleic acid, the values usually range from 2.0 to 2.2. Again, if the ratio is considerably lower, it could indicate the presence of contaminants which also absorb at 230 nm (Technical Bulletin NanoDrop Spectrophotometers).

After quantification, a 652 bp fragment of the mitochondrial *COI* gene was amplified for two samples per species using FishF1 and FishR1 primers (**Table 2.1**). The 15 µl PCR reaction included 10 X PCR buffer, 50 mM $MgCl_2$, 10 µM of each primer, 0.05 mM dNTPs, 0.625 U of Taq polymerase, 50 ng/µl DNA and Milli-Q water. The recommended PCR cycling conditions outlined in Ward *et al*. (2005) were used for amplification in the presence of negative (no template) controls and carried out in a SimpliAmp Thermal Cycler. The PCR amplicons were visualised on a 1.5 % (w/v) agarose electrophoresis gel stained with ethidium bromide (EtBr), together with a negative control, positive control and a Promega 100 bp molecular size ladder, for confirmation of successful amplification of the gene fragment (652 bp). The PCR amplicons that amplified successfully were sequenced using standard Sanger sequencing chemistry (BigDye® Terminator v.3.1 Cycle Sequencing Kit, Life Technologies, South Africa) and capillary electrophoresis performed at the DNA sequencing unit of Stellenbosch

University – the Central Analytical Facility (CAF). Sequences were manually checked, edited and trimmed in MEGA7 (Kumar *et al*. 2016). Species identification was determined by comparing sequences to the National Center for Biotechnology Information (NCBI) GenBank database and the Barcode of Life Data System (BOLD). The samples for the positive control sample list (**Table 2.3**) that amplified successfully, were used as positive control samples for the optimisation of the mini-barcoding multiplex assay.

2.2.2 Mini-barcoding multiplex assay optimisation based on positive control samples

One or two positive control samples per species with successful *COI* amplification (**Table 2.3**) were used for the mini-barcoding assay optimisation. The mini-barcoding multiplex assay (Cardeñosa *et al*. 2017) makes use of five primers, namely: VF2_tl, FishR1_tl, FishR2_tl, Shark150R and Shark474F (**Table 2.1**). This multiplex PCR leads to amplification of three mitochondrial *COI* gene fragments, with expected gene fragment sizes of ~150 bp, ~200 bp and in some instances ~650 bp (**Figure 2.1**).

Table 2.1: Mitochondrial *COI* primer pair names and sequences used to test genomic DNA amplification of control samples, as well as five mitochondrial *COI* primer names, sequences and volumes (initial concentration = 10 µM) used in the mini-barcoding multiplex PCR of 15 µl (adapted from Cardeñosa *et al*. 2017).

Primer Name	Primer Sequence (5´–3´)	Volume (µl)	References
FishF1	TCAACCAACCACAAAGACATTGGCAC	-	Ward *et al*. 2005
FishR1	TAGACTTCTGGGTGGCCAAAGAATCA	-	Ward *et al*. 2005
VF2_tl	TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC	0.9000	Ward *et al*. 2005
FishR1_tl	CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA	0.4500	Ward *et al*. 2005
FishR2_tl	CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA	0.4500	Ward *et al*. 2005
Shark150R	AAGATTACAAAAGCGTGGGC	0.2250	Fields *et al*. 2015
Shark474F	CHATTTCCCAATATCAAACACC	0.1125	Cardeñosa *et al*. 2017

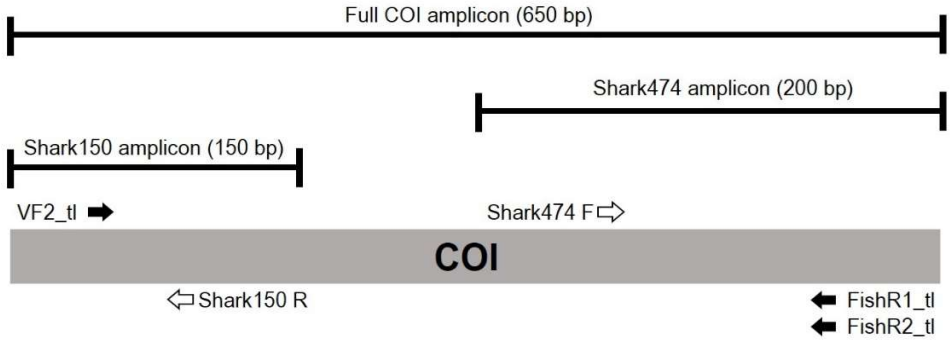


Figure 2.1: Mitochondrial *COI* gene region showing annealing sites and orientation of the five primers used in the mini-barcoding multiplex PCR, resulting in three *COI* amplicons; with solid arrows indicating universal primers. Adapted from Cardeñosa *et al*. (2017).

To make it a more cost-effective method, an adjusted 15 µl PCR reaction was used (unlike the 25 µl reaction outlined in Cardeñosa *et al*. (2017)). This included 10 X PCR buffer, 50 mM $MgCl_2$, primer mix (five primers, volumes listed in **Table 2.1**), 0.05 mM dNTPs, 0.625 U of Taq polymerase, 50 ng/µl DNA and Milli-Q water, carried out in a SimpliAmp Thermal Cycler. The recommended PCR cycling conditions of Cardeñosa *et al*. (2017) were initially used and then optimised by adjusting the annealing temperature from 52 °C to 54 °C. The PCR amplicons were visualised on a 3 % (w/v) agarose electrophoresis gel for confirmation of successful amplification of the three mitochondrial *COI* gene fragments (150 bp, 200 bp and 650 bp). For comparison purposes, in a subset of nine case study samples, the *COI* gene region (652 bp) was first amplified using universal FishF1 and FishR1 primers (**Table 2.1**) and then using the optimised mini-barcoding multiplex PCR and conditions. For standard Sanger sequencing chemistry (BigDye® Terminator v.3.1 Cycle Sequencing Kit, Life Technologies, South Africa), the M13F primer was used when the 150 bp amplicon was present, M13R primer for the 200 bp amplicon and both M13F and M13R primers were used when the 650 bp amplicon was present (primers shown in **Table 2.2**). Capillary electrophoresis was performed at CAF and sequence analyses was then performed for species identification (as mentioned in section 2.2.1).

Table 2.2: Mitochondrial *COI* primer names and sequences, used in Sanger sequencing reactions, for the resulting amplicons of the mini-barcoding assay.

Primer name	Region	Primer sequence (5´–3´)	Reference
M13F	*COI*	TGTAAAACGACGGCCAGT	Strüder-Kypke and Lynn 2010
M13R	*COI*	CAGGAAACAGCTATGAC	Strüder-Kypke and Lynn 2010

2.2.3 Application of traditional barcoding and optimised mini-barcoding assay to case studies involving southern African marine species

All shark fins received from case studies were hydrated in a saline solution (2 % NaCl) after which fin clip samples were taken and stored in 99 % ethanol. Total gDNA was extracted from fin clip samples using an adjusted CTAB DNA extraction protocol (Sambrook and Russell 2001). DNA quantification (ng/µl) and quality was assessed using a NanoDrop Spectrophotometer and concentrations adjusted to 50 ng/µl. Additionally, DNA was visualised on a 1.5 % (w/v) agarose electrophoresis gel to determine if DNA was intact. This is especially important for confiscated fins as these could be from different stages of processing, from a dried to a chemically treated state. Thereafter the optimised mini-barcoding multiplex assay (adapted from Cardeñosa *et al*. (2017)) was employed to amplify the three gene regions (150 bp, 200 bp and in some instances 650 bp). This was followed by a 3 % (w/v) agarose electrophoresis gel to assess successful amplification of any of the three gene fragments.

Fish products were purchased from a variety of retail outlets in the Western Cape, South Africa, including chain stores and fish takeaway shops (n = 7). The fish products included hake fish cakes, hake portions, fish fingers and salt fish – shown in **Figure 2.2 a** and **Figure 2.2 b**.

a **b**

Figure 2.2: **(a)** Fish cake sample during the process of DNA extraction – isolating fish flakes and **(b)** "Salt fish" sample during the process of DNA extraction.

Fish flakes were isolated from the respective products (~0.5 cm^2), being careful not to include the bread crumbs, potato and rice fillings; and samples were stored in separate tubes at -20 °C. Thereafter, total gDNA was extracted from fish flake samples and DNA quantification (ng/µl) and quality (absorbance ratio: 260/280 and 260/230) was then determined using a NanoDrop Spectrophotometer and visualised on a 1.5 % (w/v) agarose electrophoresis gel, all as mentioned previously. Amplification of DNA from fish samples was tested using a PCR containing primers FishF1 and FishR1 (**Table 2.1**), using reagents and cycling conditions outlined above (Ward *et al*. 2005), to amplify the *COI* barcoding gene region (652 bp). The PCR amplicons were visualised on a 1.5 % (w/v) agarose electrophoresis gel, for confirmation of successful amplification of the gene fragment (652 bp).

Polymerase chain reaction amplicons for both confiscated shark fin samples and fish product samples were sequenced using standard Sanger sequencing chemistry (BigDye® Terminator v.3.1 Cycle Sequencing Kit, Life Technologies, South Africa) and capillary electrophoresis performed at CAF (DNA sequencing facility, Stellenbosch University). Sequences were manually checked, edited and trimmed in MEGA7 (Kumar *et al*. 2016) and species identification determined by comparing sequences to the NCBI GenBank database and BOLD to the lowest taxonomic category as possible. A 98 % match at least is used for reliable species identification (Barbuto *et al*. 2010); therefore, accurate species identification was based on 98–100 % sequence similarity.

2.3 Results

2.3.1 Species control list, multiplex optimisation and a comparison between barcoding methods

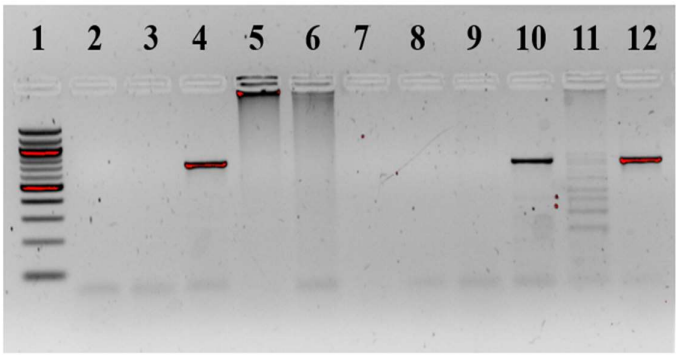
The positive control sample list comprises 26 species (**Table 2.3**) and includes mainly southern African commercially exploited shark and ray species. Importantly, the main species affected by the demersal shark trade in southern Africa were included, namely: *Mustelus mustelus*, *Mustelus palumbes*, *Galeorhinus galeus*, *Carcharhinus brachyurus* and *Carcharhinus obscurus* (da Silva and Bürgener 2007). Also, two hammerhead species *Sphyrna lewini* and *Sphyrna zygaena* that are both CITES-listed were included.

Table 2.3: List of 26 species used for the positive control sample list. Species identification confirmed by DNA barcoding, using genomic DNA and the Fish1 primer pair, amplifying a 652 bp region of the mitochondrial *COI* gene.

Scientific name	Common name	Scientific name	Common name
Carcharhinus limbatus	blacktip	*Carcharhinus leucas*	bull
Sphyrna lewini	scalloped hammerhead	*Mustelus palumbes*	whitespotted smoothhound
Sphyrna zygaena	smooth hammerhead	*Mustelus mustelus*	common smoothhound
Carcharhinus brachyurus	copper	*Mustelus mosis*	arabian smoothhound
Galeorhinus galeus	tope	*Scylliogaleus quecketti*	flapnose houndshark
Carcharhinus obscurus	dusky	*Triakis megalopterus*	spotted gully
Carcharhinus plumbeus	sandbar	*Etmopterus sculptus*	sculpted lanternshark
Gymnura natalensis	diamond ray	*Scyliorhinus capensis*	yellow spotted catshark
Mylobatis aquila	common eagle ray	*Holohalaelurus regani*	izak catshark
Rostroraja alba	bottlenose skate	*Poroderma africanum*	pyjama catshark
Carcharias taurus	ragged-tooth	*Haploblepharus pictus*	dark shyshark
Carcharhinus brevipinna	spinner	*Haploblepharus edwardsii*	puffadder shyshark
Prionace glauca	blue	*Haploblepharus fuscus*	brown shyshark

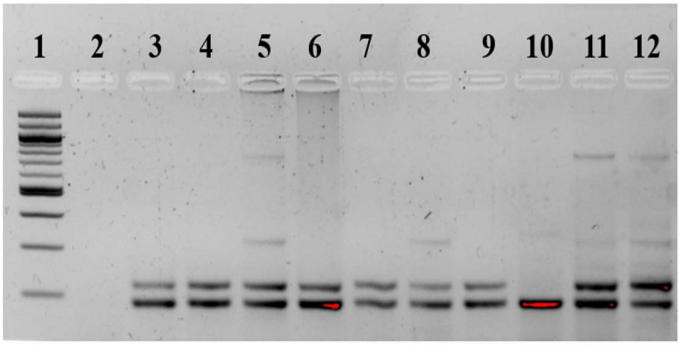
The multiplex PCR protocol was optimised with the optimal T_A at 54 °C (not 52 °C as outlined in Cardeñosa *et al*. (2017)). This increases the stringency of primer annealing, thus also making the PCR amplification more specific, and decreasing the chance of unspecific PCR products (Malhotra *et al*. 1998). All positive control samples (**Table 2.3**) could be amplified for appropriate regions using the mini-barcoding multiplex assay resulting in 150 bp, 200 bp and, in some instances, 650 bp *COI* PCR fragments.

In the case study where a subset of nine fin clip samples were tested using traditional *COI* barcoding amplification (using FishF1 and FishR1 primers; **Table 2.1**), only two of the samples (2/9) amplified successfully for this gene region (**Figure 2.3**). However, using the mini-barcoding multiplex assay, all samples (9/9) amplified successfully for the 150 bp gene fragment, most samples for the 200 bp and some for the 650 bp fragment (**Figure 2.4**). The aforementioned results showed the limitations of using only the traditional *COI* barcoding primers (Fish1 primers; **Table 2.1**) when dealing with confiscated shark fins that are possibly dried or chemically treated. This warrants the inclusion of smaller *COI* gene regions for species identification in forensic cases involving processed shark fins, making the mini-barcoding assay more ideal. This optimised mini-barcoding assay could be applied to forensic case studies involving confiscated shark fins.



Lane	Description
1	100 bp ladder
2	Negative control
3 – 11	Samples 1 – 9
12	Positive control

Figure 2.3: 2 % (w/v) agarose gel electrophoresis image of *COI* PCR products (652 bp), using the Fish1 barcoding primer pair and genomic DNA from nine case study samples. Samples in lanes 4 and 10 show positive amplification of the 650 bp region.



Lane	Description
1	100 bp ladder
2	Negative control
3 – 11	Samples 1 – 9
12	Positive control

Figure 2.4: 3 % (w/v) agarose gel electrophoresis image of PCR products using the mini-barcoding multiplex PCR, indicating three *COI* gene fragments (150 bp, 200 bp and 650 bp) using genomic DNA from nine case study samples. All samples show positive amplification of the 150 bp band and all samples except for the sample in lane 10, show positive amplification of the 200 bp band.

2.3.2 Possible mislabelled fish products from retail outlets in the Western Cape, South Africa

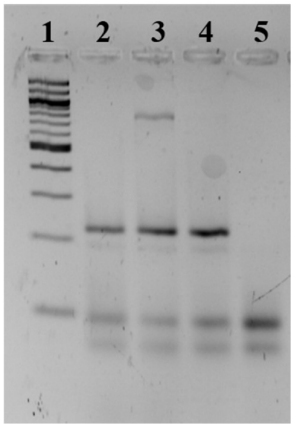
Fish products that were purchased consisted of hake fish cakes, hake portions, hake fish fingers and salt fish. The seven samples tested using *COI* sequencing (652 bp) and identification were compared to the species names labelled on each product. The results are shown in **Table 2.4** and consisted of deep-water Cape hake *Merluccius paradoxus*, shallow-water Cape hake *Merluccius capensis* and snoek *Thyrsites atun* – all with a species identification match > 98 %. None of the seven samples tested were incorrectly labelled. One sample was, however, labelled ambiguously as "salt fish" with no official packaging, while two fish cake samples had "and/or" on the label (**Table 2.4** – hake and/or pollock as well as *Merluccius paradoxus* and/or *capensis*) and three of the labels only provided the genus name for all hake species (*Merluccius* spp.). Interestingly, only one out of the seven samples product's label provided the full and correct species name.

Table 2.4: Seven fish products identified to the species-level, from retail outlets in the Western Cape, South Africa. The product label name as indicated on the product (showing % composition), species identification based on DNA barcoding (using Fish1 primers), BOLD identification number and match percentage identity (%) are indicated.

Fish product type	Product label name (% composition)	Species identification	BOLD ID no. (%)
Fish cake (Brand 1)	34 % *Merluccius* spp.	*Merluccius paradoxus* (deep-water Cape hake)	BOLD:AAC4936 (100)
Fish cake (Brand 2)	46 % *Merluccius* spp.	*Merluccius capensis* (shallow-water Cape hake)	BOLD:AAC3762 (98.71)
Fish fingers (Brand 2)	65 % *Merluccius* spp.	*Merluccius capensis* (shallow-water Cape hake)	BOLD:AAC3762 (100)
Fish cake (Brand 3)	Hake and/or pollock	*Merluccius paradoxus* (deep-water Cape hake)	BOLD:AAC4936 (100)
Fish portion (Brand 4)	*Merluccius paradoxus* and/or *capensis*	*Merluccius paradoxus* (deep-water Cape hake)	BOLD:AAC4936 (100)
Fish cake (Brand 5)	58 % *Merluccius capensis*	*Merluccius capensis* (shallow-water Cape hake)	BOLD:AAC3762 (100)
Salt fish (Brand 6)	Salt fish	*Thyrsites atun* (snoek)	BOLD:AAB5033 (100)

2.3.3 Southern African shark forensic case studies: Confiscated shark fins for possible illegal trade

For the first case study, juvenile specimens were confiscated from an illegal fishing vessel by the Department of Agriculture, Forestry and Fisheries (DAFF; South Africa, Government department) during port inspections at the Cape Town Harbour, South Africa. The location of exactly where these sharks were fished is unknown and samples were morphologically identified as common smoothhound *Mustelus mustelus*. Results from the mini-barcoding assay indicated that all samples amplified for the 200 bp band, some samples show a weaker amplified 150 bp band and one sample a weaker 650 bp band (a subset of samples shown in **Figure 2.5**).



Lane	Description
1	100 bp ladder
2 – 4	Samples 1 – 3
5	Negative control

Figure 2.5: 3 % (w/v) agarose gel electrophoresis image of PCR products using the mini-barcoding multiplex PCR, using genomic DNA from three case study samples from shark fins confiscated at Cape Town Harbour, South Africa. All three samples show amplification of the 200 bp *COI* gene fragment.

After comparing specimen sequences to reference sequences on BOLD and NCBI GenBank Database, it was concluded that for the 200 bp sequences, species-level identification could not be made but confirmed them to be from the genus *Mustelus*. Top hits consisted of: *Mustelus manazo*, *Mustelus asterias* and *Mustelus palumbes*; however, *M. manazo* and *M. asterias* species do not occur in southern Africa waters so it is more likely the specimens are whitespotted smoothhound *M. palumbes*. It was in fact concluded that the specimens are *M. palumbes*, confirmed by the *COI* sequences aligned to both *M. mustelus* and *M. palumbes* reference sequences (with a percent identity of 92.56 % and 100 % respectively). Subsequently, this indicates that the particular 200 bp fragment of the *COI* gene is not sufficient to identify *Mustelus* spp. to the species-level. Most notably, when using this mini-barcoding assay for species identification of closely related species such as *Mustelus* spp., either the full gene fragment region (650 bp) or the 150 bp fragment should be used in addition to the 200 bp sequence. The multiplex allows for these alternate fragments to be amplified and this can be taken into consideration for future case studies.

A second case study involved the confiscation of shark fins at OR Tambo International Airport (Johannesburg, South Africa), which were believed to be in transit from Mozambique to Hong Kong. They were declared as blue shark fins and later suspected to be grey reef shark *Carcharhinus amblyrhynchos* as well as hammerhead shark species (*Sphyrna* spp.), based on morphological

identification. The Department of Environmental Affairs (DEA; South African government department) requested that the shark fin samples be analysed genetically to determine the species of origin. A subset of ten fins were analysed and based on sequence analysis, a total of six shark species were identified as the following: sandbar shark *Carcharhinus plumbeus*, bull shark *Carcharhinus leucas*, graceful shark *Carcharhinus amblyrhynchoides*, blacktip shark *Carcharhinus limbatus*, spinner shark *Carcharhinus brevipinna* and pigeye shark *Carcharhinus amboinensis* (**Table 2.5**).

Table 2.5: Ten samples from shark fins confiscated at OR Tambo International Airport, South Africa; species-level identification was performed using the mini-barcoding multiplex assay. The BOLD identification number, match percentage identity (%) and IUCN Red List Status is also indicated. IUCN Red List Categories: Vulnerable – high risk of extinction in the wild, Near Threatened – likely to qualify for a threatened category in the near future, Data Deficient – inadequate information for an assessment.

Sample No.	Most similar species	BOLD ID no. (%)	IUCN Red List Status
9	*Carcharhinus plumbeus* (sandbar)	BOLD:AAA4896 (100)	Vulnerable
1, 2, 7	*Carcharhinus leucas* (bull)	BOLD:AAA6060 (100)	Near Threatened
4, 10	*Carcharhinus amblyrhynchoides* (graceful)	BOLD:AAA5251 (100)	Near Threatened
5, 8	*Carcharhinus limbatus* (blacktip)	BOLD:AAA5251 (100)	Near Threatened
3	*Carcharhinus brevipinna* (spinner)	BOLD:AAA3388 (99.26)	Near Threatened
6	*Carcharhinus amboinensis* (pigeye/java)	BOLD:ACF2385 (100)	Data Deficient

All samples showed a > 98 % sequence identity and are therefore considered reliable for species identification (Barbuto *et al*. 2010). The six species identified are all relatively large pelagic shark species (except *C. limbatus*), all belonging to the genus *Carcharhinus*. The large sizes of the species identified is more likely due to the susceptibility of the larger-sized shark to be caught by the longline gear, rather than by deliberate targeting of specific species. An attempt to delineate the identified species based on their species range was also performed and indicated that some species could be mapped to their geographical distributions. For example, unambiguously identified species includes those that were mapped to the South-West Indian region, suggesting that these sharks were likely caught from Mozambican waters. Based on the IUCN Red List of Threatened Species, these species range from

Vulnerable to Near Threatened, also including those categorised as Data Deficient. Three of these shark species, namely bull shark *C. leucas*, blacktip shark *C. limbatus* and graceful shark *C. amblyrhynchoides* were represented more than once – which is an indication of the targeting of larger shark species. For example, *C. leucas* can grow to a total length of 3.4 m (Compagno *et al*. 2005), and are thus targeted for their fins.

A third case study involved 109 pieces of shark fins confiscated from two locations (A and B) along the southern African coast. These fin pieces were cut into irregular shapes and consisted of different colours and forms (**Figure 2.6**). Samples were extremely dried out, possibly treated with chemicals, and based on morphological identification; some were suspected to be from hammerhead shark species (*Sphyrna* spp.). A subset of 29 samples were selected for species identification: 21 samples from the first location (Location A) and eight samples from the second location (Location B).

Figure 2.6: Shark fin samples confiscated from two locations (Location A and B) along the southern African coastline that consisted of different shapes, sizes and forms. Species-level identification was performed using the optimised mini-barcoding multiplex assay.

The 150 bp *COI* gene region amplified for most samples and only a small portion of samples amplified for the 200 bp *COI* gene region. This could be due to degradation of the DNA owing to the processed state of the shark fins. Based on sequences from the 150 bp and 200 bp *COI* gene regions, eighteen samples were positively identified to the species-level (**Table 2.6**), consisting of eight different shark species, with all samples showing a species identity of > 98 %. The remaining eleven samples could not be identified to the species-level; however, the genus *Carcharhinus* was confirmed for these samples. The IUCN Red List Status of the identified species include the following categories: Critically Endangered (*Rhynchobatus djiddensis*), Endangered (*Sphyrna lewini* and *Isurus oxyrinchus*),

Vulnerable (*Carcharhinus albimarginatus*), Near Threatened (*Carcharhinus amblyrhynchos*, *Carcharhinus brachyurus* and *Galeocerdo cuvier*) and Data Deficient (*Carcharhinus amboinensis*). There are three duplicate species, namely scalloped hammerhead *Sphyrna lewini*, shortfin mako *Isurus oxyrinchus* and whitespotted wedgefish *Rhynchobatus djiddensis*, which could be an indication for the targeting of species containing fins with a higher market value.

Table 2.6: Eighteen samples from shark fins confiscated from two locations (A and B); species-level identification was performed using the optimised mini-barcoding multiplex assay. The BOLD identification number, match percentage identity (%) and IUCN Red List Status is also indicated. IUCN Red List Categories: Critically Endangered – extremely high risk of extinction in the wild, Endangered – very high risk of extinction in the wild, Vulnerable – high risk of extinction in the wild, Near Threatened – likely to qualify for a threatened category in the near future, Data Deficient – inadequate information for an assessment.

Location A			
Sample No.	**Most similar species**	**BOLD ID no. (%)**	**IUCN Red List Status**
1, 11, 12	*Rhynchobatus djiddensis* (whitespotted wedgefish)	BOLD:AAC4065 (100)	Critically Endangered
4, 16, 17	*Sphyrna lewini* (scalloped hammerhead)	BOLD:AAA2402 (99.26)	Endangered
3	*Carcharhinus albimarginatus* (silvertip)	BOLD:AAA6605 (99.26)	Vulnerable
10	*Carcharhinus amblyrhynchos* (grey reef)	BOLD:AAA6604 (100)	Near threatened
15	*Carcharhinus brachyurus* (copper)	BOLD:ACE6212 (100)	Near threatened
18	*Galeocerdo cuvier* (tiger)	BOLD:AAA2006 (99.42)	Near threatened
2	*Carcharhinus amboinensis* (pigeye/java)	BOLD:ACF2385 (99.24)	Data Deficient
Location B			
Sample No.	**Most similar species**	**BOLD ID no. (%)**	**IUCN Red List Status**
5, 6, 8, 9	*Sphyrna lewini* (scalloped hammerhead)	BOLD:AAA2402 (99.26)	Endangered
7, 13, 14	*Isurus oxyrinchus* (shortfin mako)	BOLD:AAA4689 (99.7)	Endangered

2.4 Discussion

2.4.1 Mini-barcoding assay optimisation for southern African commercially exploited shark species

Overall the limitations of only using the traditional *COI* barcoding primers for shark forensic species identification purposes are highlighted in the current study. This is possibly due to the majority of confiscated shark fins being in a processed state (dried or chemically treated). When using the *COI* barcoding Fish1 primers on these shark fin samples, only a 22 % (2/9) species identification success rate was obtained, whereas the mini-barcoding assay showed a 100 % success rate on the exact same samples. The mini-barcoding assay allows for shorter (150 bp and 200 bp) *COI* gene fragments to be amplified, in addition to the full *COI* barcoding gene region. Therefore, this makes the mini-barcoding assay ideal for fin samples and possibly other shark products (such as salted shark meat) that most likely only contain DNA in a degraded state. The latter is known to result in reduced amplification and sequencing success for genetic identification (Cardeñosa *et al*. 2017). The mini-barcoding assay was optimised for commercially exploited shark species more specific to southern Africa, and to be more cost-effective (reduced reagent volume). Most importantly, the assay can be used to identify shark fins from the five most exploited southern African shark species: common smoothhound *M. mustelus*, whitespotted smoothhound *M. palumbes*, tope shark *G. galeus*, copper shark *C. brachyurus* and dusky shark *C. obscurus* – in order of commercial importance (da Silva and Bürgener 2007). In addition, the assay can also be used to identify shark fins and shark products intended for the international trade. In particular, the assay can identify CITES-listed sharks including scalloped hammerhead *S. lewini* and smooth hammerhead *S. zygaena*, which are relatively important among current fin imports to Hong Kong (Cardeñosa *et al*. 2018a). Therefore, this optimised mini-barcoding multiplex assay can be applied to wildlife case studies involving forensic species identification of shark fin and shark products commonly found in trade, thus allowing the detection of potential illicit trade in threatened species, in southern Africa as well as internationally.

2.4.2 Fish products from retail outlets in South Africa with misleading labels

The matching of fish product labels to species identification, based on *COI* sequences, allowed the detection of possible cases of mislabelled, misleading or ambiguously labelled products. Only one out of seven products had the full and correct species name included on the label. Four of the product's ingredients stated the genus name for hake (*Merluccius* spp.) and one of the product labels stated broad main ingredient names such as "hake and/or pollock". Hake (*Merluccius* spp.) and pollock (*Pollachius* spp.) are both white flesh fish, belonging to the same order, *Gadiformes*. There are sixteen species of hake (*Merluccius* spp.) with two species found in southern Africa, namely *M. capensis* and *M. paradoxus* (Rademeyer *et al*. 2008). However, pollock (*Pollachius* spp.) is distributed in the North Pacific, northeastern Atlantic (Norway and Iceland) and in the western Atlantic (Iceland and Southwest Greenland) (FAO 1990); and therefore, would have to be imported and sold in South Africa. A potential issue with including only the genus name (for example in the case *Merluccius* spp.) or general fish names (for example in the case of "hake and/or pollock") on the product label would be when the product label states "product of South Africa". This implies that the main ingredient of the product is derived from South Africa, and would be an issue when neither of the two southern African hake species *M. capensis* and *M. paradoxus* is present. If it is in fact *Pollachius* spp., this goes against labelling regulations of South Africa (DoH 2010) and the product could be considered mislabelled. Thus, only having the genus name or general names leaves it open to a possible case of seafood fraud. Previously, there has been several cases in South Africa of the possible mismatch between fish product labels and molecular species identification. In one case, *COI* sequencing revealed a 100 % sequence similarity to the imported species *Merluccius productus* (North Pacific hake). In another case, demonstrating potential misleading labelling, a regularly consumed fish commonly named "yellowtail" is expected to be referred to as *Seriola lalandi* (yellowtail amberjack). However, five retail samples labelled as "yellowtail" showed a 100 % sequence similarity to *Seriola quinqueradiata* (Japanese amberjack), which is not from South Africa and most likely imported from Asian countries. Thus, these cases not only involved misrepresentation at the species-level, but also at the country-of-origin level (Cawthorn *et al*. 2011).

One of the samples tested in the current study was sold as "salt fish" which is fleshy white fish, typically cod fish (*Gadus* spp.), preserved by salt-curing and drying. Salted cod is also known as "bacalao" in the European and South American market (Kristjansson 2013). Other common fish that are salt-cured beside cod (*Gadus* spp.) include pollock (also *Gadus* spp.), snapper (*Lutjanidae* spp.), and most notably, shark meat (Nelson 2018). Interestingly for this case, snoek (*Thyrsites atun*) was found to be the source of "salt fish" with no indication at the time of purchase what species it was (**Figure 2.2 b**). The fact that shark meat is used for this form of fish and no label is indicated at the time of purchase makes it a risk factor for consumers, in terms of making a decision about potentially eating shark or unknown fish species, and therefore a risk for possible fish allergies and unknown mercury intake. Methylmercury is accumulated by fish and marine mammals, with highest concentrations attained in large predatory species at the top of the aquatic food chain (WHO 2000). In a study by McKinney *et al*. (2016), mercury concentrations for a high number of southern African fish species were above the regulatory guidelines for fish health and safe human consumption. Thus, causing concern not only for shark health, but also consumption by humans of shark meat from South Africa (McKinney *et al*. 2016). Fish allergies are a pathophysiological, IgE-mediated immune response to specific fish proteins, whereby IgE (Immunoglobulin E) are antibodies produced by the body's immune system that respond to an allergy causing a reaction (Kuehn *et al*. 2014). The reaction can be a variety of conditions, such as: oral allergy syndrome, abdominal pain, diarrhoea, asthma, and in severe cases, life-threatening anaphylaxis (Helbling *et al*. 1999; Bock *et al*. 2001). Specifically, with regards to fish allergies to hake and snoek, a study was done in South Africa based on a questionnaire of 105 subjects with a history of seafood allergy. Results showed that the most common bony fish species causing IgE-mediated allergic reactions was hake (24.8 %) (Zinn *et al*. 1997; Sharp and Lopata 2013). Subsequently, ten fish-allergic consumers were assessed based on five fish species and results indicated that pilchard displayed the strongest IgE reactivity, followed by anchovy, snoek, hake and yellowtail (Beale *et al*. 2009). These studies indicate that hake and snoek are significant contributors to fish allergic reactions, and it is therefore important for precise labels on fish products.

The current case study of a few fish products indicates some important factors to consider when purchasing these products in South Africa. It shows that not all fish products' main ingredients are from South Africa and ambiguous labelling is common, with an instance where "salt fish" can be any white-flesh fish or even shark meat. The study therefore confirms what was previously found: that misrepresentation at the species-level as well as at the country-of-origin level does occur within South African fish products (Cawthorn *et al*. 2011). These factors increase the possibility of consuming shark species and other fish species unknowingly, and thus increase the risk of unsafe mercury intake, fish allergy and allergic reactions.

2.4.3 Case studies involving confiscated shark fins in southern Africa and the implications thereof

Shark fins confiscated from Cape Town Harbour were morphologically identified as *Mustelus mustelus*; however, based on *COI* sequencing (200 bp fragment) they are most likely *Mustelus palumbes*. A 100 % match on BOLD could not be obtained based on only the 200 bp sequence. However, for future case studies involving closely related species such as *Mustelus* spp., additional *COI* gene region sequences should be included if possible. Based on an alignment of the *COI* sequences, specimens are most likely *M. palumbes* as opposed to *M. mustelus*, highlighting morphological misidentification issues. The misidentification in this case could be attributed to the fact that samples were from juvenile specimens, and some identification features are not yet developed or visible, therefore making morphological identification not as accurate. *Mustelus mustelus* is typically unspotted but can have sparse black spots or blotches (but no very dark spots or dark bars) on the dorsal surface instead of the numerous small white spots characteristic for *M. palumbes* (Compagno 1984; Farrell *et al*. 2009). Specifically, *M. palumbes* is currently classified as Data Deficient on the IUCN Red List of Threatened Species and is endemic to southern Africa (Namibia, South Africa and Mozambique) (Smale 2006). *Mustelus palumbes* is predominantly caught in trawl fisheries off both the south and west coasts of South Africa. The demersal shark longline fishery operates in southern African coastal waters ranging from the West Coast Orange River to the East Coast Kei River (DAFF 2012). However, recent

data from South Africa suggests that trawl catches of *Mustelus* spp. taken by demersal cruises are declining. Estimated landings from 2010, 2011 and 2012 were 408 tonnes, 175 tonnes and 88 tonnes of shark respectively (DAFF 2012). This apparent drop in landed catches may be attributed to the combined effects of a decline in abundance, effort displacement towards more profitable fisheries or target availability. Common species caught from the demersal shark longline fishery include both *M. mustelus* and *M. palumbes* (DAFF 2012). Since then, in 2013, the South African National Plan of Action for sharks (NPOA-Sharks) was finalised and provided information regarding management, monitoring, research and enforcement related to shark fishing and trade of shark products in South Africa. According to the implementation reports, the most remarkable progress has been made with regards to *optimum use* (e.g. health risk associated with shark meat consumption) and the *classification and assessment of species* (e.g. taxonomic revision and genetics research of South African shark species). Currently, the South African NPOA-Sharks is being updated with intended completion at the end of 2019 (da Silva *et al*. 2018), thus improving management efforts of chondrichthyan fisheries in South Africa.

Shark fins confiscated from OR Tambo International Airport in Johannesburg, South Africa were all identified to the species-level using the mini-barcoding approach. Six species were identified from the *Carcharhinus* genus and not what they were suspected to be, which were either grey reef shark *Carcharhinus amblyrhynchos* or hammerhead sharks (*Sphyrna* spp.). In a recent study, during 2014–2015, bull shark *Carcharhinus leucas*, blacktip shark *Carcharhinus limbatus* and spinner shark *Carcharhinus brevipinna* were three of eight species that each comprised more than 1 % of the fin trimmings from an assessment of a retail market (Sheung Wan and Sai Ying Pun fin market) in Hong Kong (Fields *et al*. 2018). During the same study, graceful shark *Carcharhinus amblyrhynchoides*, pigeye shark *Carcharhinus amboinensis* and sandbar shark *Carcharhinus plumbeus* are also mentioned as being sought after, specifically for the shark fin trade. A few *C. amblyrhynchoides* samples (0.13 %) were identified from the 2014–2015 trimmings, while 54 samples of *C. amboinensis* (1.13 %) were identified in the study (Fields *et al*. 2018). This is concerning as *C. amboinensis* seems to be highly

structured genetically, making coastal populations even more vulnerable to localised overexploitation (Chapman *et al*. 2015). Previously, the sandbar shark *C. plumbeus* was commonly found to be auctioned for their fins, making up 2–3 % of the fins auctioned in Hong Kong (Clarke *et al*. 2006a). However, in the recent study by Fields *et al*. (2018), *C. plumbeus* was rarely encountered where only eleven samples were identified from the 2014–2015 trimmings (0.23 %). Fisheries located on the coast of western Australia and the Atlantic coast of the United States were supplying large amounts of *C. plumbeus* from 1999–2001. Subsequently, significant population declines of *C. plumbeus* led to large reductions in catch limits (McAuley and Rowland 2012). Thus, the current study also clearly suggests that the above-mentioned species are of some importance for the shark fin trade and market in Hong Kong. These results suggest that policies aimed at mitigating shark species extinction vulnerability needs to be comprehensive and co-ordinated at a global level.

With regards to shark fins confiscated elsewhere in southern Africa, only eighteen of the 29 shark fin samples tested could be identified to the species-level (**Table 2.6**); and a total of eight shark species were identified. At the time of the case study, approximately 40 % of the samples tested were from CITES-listed species. Of high concern is that based on updated and current (2019) CITES listings, over 70 % of the samples tested were from CITES-listed shark species including: the shortfin mako *Isurus oxyrinchus*, whitespotted wedgefish *Rhynchobatus djiddensis* and the scalloped hammerhead *Sphyrna lewini*. Hammerhead species (*Sphyrna* spp.) are one of the top sources for shark fins as they have the best quality fin needles (ceratotrichia) for consumption, and have a high commercial value in the Asian shark fin trade (Abercrombie *et al*. 2005). *Sphyrna lewini* is considered to be experiencing the most severe population declines (Ferretti *et al*. 2008). Specifically, in South Africa, a decline of 64 % for S. *lewini* populations over a 25-year period (1978–2003) was observed (Dudley and Simpfendorfer 2006). The shortfin mako *I. oxyrinchus*, was also identified in a large proportion of the samples. This is concerning for an Endangered species, as a recent study showed fishing mortality rates were well above those previously reported for the species (Byrne *et al*. 2017). According to the Fields *et al*. (2018) study, 2.77 % of samples from the fin market (Sheung Wan and Sai Ying Pun) consisted

of *I. oxyrinchus* and CITES recently listed this species in Appendix II (CITES 2019). Also noteworthy, is that 17 % (3/18) of the samples in the current case study were identified as whitespotted wedgefish, *Rhynchobatus djiddensis*. Broadly, *R. djiddensis* belongs to the shark family of wedgefishes, which are large benthopelagic shark-like rays (Giles *et al*. 2016). *Rhynchobatus djiddensis* is exploited by fisheries driven by the high value of their fins in international trade, and declines have been noted throughout their range (Moore 2017; Jabado 2018). A recent trend shows wedgefishes' fins are becoming more common in the shark fin trade (Fields *et al*. 2018). More regionally, declines of *R. djiddensis* have been observed off Madagascar and Mozambique, where these locations were previously reportedly abundant in this species (Pierce *et al*. 2008; Hopkins 2011). These declines are also linked to the highly desirable fins of wedgefishes in the international shark fin trade; their fins reaching some of the highest prices based on their size (Rose and McLoughlin 2001; Clarke *et al*. 2006a). In South Africa, *R. djiddensis* is caught as bycatch by demersal prawn trawlers operating on the Tugela Bank (located off central KwaZulu-Natal). Most caught are alive and released, although subsequent survival is not known (Fennessy 1994). Heavier exploitation of *R. djiddensis* used to occur, for example, in Tanzania through bottom-set gillnets, prawn trawlers and possibly also by spearfishermen (Barnett 1997). Additionally, the targeting of wedgefishes has been noted by foreign vessels off western Africa (offshore Mozambique, Tanzania and Madagascar) (Kyne *et al*. 2019b). A report of illegal fishing by foreign vessels in Mozambican waters included a seisure of an entire cargo consisting of mostly *R. djiddensis* (Dudley and Cavanagh 2006). Recently, wedgefishes have been noted as one of the shark families showing severe population declines globally, resulting in 15 of 16 species (94 %) assessed as Critically Endangered on the IUCN Red List of Threatened Species (Kyne *et al*. 2019b), including *R. djiddensis* (Kyne *et al*. 2019a). Subsequently, *R. djiddensis* is one of the wedgefish species also recently included in Appendix II (CITES 2019). Thus, for this species the problem does not seem to be within South Africa where *R. djiddensis* is protected (Kyne *et al*. 2019b), but rather in neighbouring countries where the species is under severe threat of exploitation. The remaining eleven samples that could not be identified to the species-level for this case study, were at least all identified to the genus-level (*Carcharhinus* spp.). From this specific case study, the DNA from these shark fin samples was degraded

due to samples being dried and possibly treated with chemicals, which was visibly evident upon inspection. Most likely species identification matches (100 %) included: spinner shark *Carcharhinus brevipinna*, blacktip shark *Carcharhinus limbatus*, graceful shark *Carcharhinus amblyrhynchoides*, sandbar shark *Carcharhinus plumbeus* and dusky shark *Carcharhinus obscurus*. All of these shark species (except *C. obscurus*) have been observed in the previous case study and are therefore suggested to be common in trade. These are all relatively large species, indicating the targeting of these larger shark species.

In terms of the different locations, for Location A there is a greater diversity of species, where seven different shark species were identified. Two of these species were represented more than once, namely scalloped hammerhead *Sphyrna lewini* and whitespotted wedgefish *Rhynchobatus djiddensis*, while fourteen samples identified as belonging to the *Carcharhinus* genus. This could signify the targeting of larger shark species, as well as shark species with higher fin value. For Location B, only two species were identified and for multiple samples, the scalloped hammerhead *Sphyrna lewini* and shortfin mako *Isurus oxyrinchus*, and are currently both CITES-listed. This could indicate the targeting of these specific species and are also identified as being common in trade due to their high fin value (Abercrombie *et al*. 2005; Fields *et al*. 2018). Overall, results of this case study indicate the continuous exploitation of CITES-listed, as well as shark species classified as Critically Endangered – and consequently their fins are being sold for illegal trade. Additionally, the fact that most of these fin samples were disguised into smaller pieces highlights the importance for molecular species identification, for law enforcement of illicit shark fin trade, in addition to visual identification of fins.

In summary, the above-mentioned case studies involving confiscated shark fins indicate that the mini-barcoding multiplex assay for closely related species (such as *Mustelus* spp. and some *Carcharhinus* spp.) is not always successful in identification to the species-level and that alternate *COI* gene fragments should also be analysed. Based on the results, it is also evident that South Africa possibly acts as an intermediate transportation zone (for example, between other Western Indian Ocean countries and Hong Kong in this particular case) for the exportation of shark fins. Lastly, it confirms

that larger pelagic shark species are the main species targeted due to their larger fin size (*Carcharhinus* spp.) as well as CITES-listed and endangered shark species (*Sphyrna lewini*, *Isurus oxyrinchus* and *Rhynchobatus djiddensis*).

2.5 Conclusion

In conclusion, this chapter reports on the application of a traditional barcoding method to a seafood case study as well as the optimisation of a mini-barcoding multiplex assay and its application to shark forensic case studies. Additionally, these two methods are compared for success in species identification – the barcoding method vs. the mini-barcoding assay, in the context of confiscated shark fins. In this context, the mini-barcoding assay proved to be more successful, with 100 % success rate compared to only 22 % for the traditional barcoding method. It was further revealed that there is some level of mislabelling and seafood fraud of fish products in the Western Cape, South Africa. Of most concern, based on case studies, is that a large percentage of confiscated shark fins in southern Africa were confirmed to be from CITES-listed and endangered species, possibly for illicit trade. The targeting and exploitation of larger shark species for trade (*Carcharhinus* spp.) was also apparent. Finally, it shows that molecular tools, more specifically the mini-barcoding multiplex assay approach, is especially useful with cases involving shark fin specimens that are in a processed state, or when visual identification is disguised in some way. Thus, this optimised assay can be applied and used as an additional tool for the enforcement and monitoring of shark species at regional and international levels, specifically identifying highly traded and threatened shark species.

CHAPTER 3

Molecular forensics: Optimisation of a High Resolution Melting (HRM) assay for southern African exploited shark species

Abstract

In South African fishery operations, major concerns are the high volumes of shark species landed, in addition to frequent species misidentification. The processing of sharks at sea further impedes identification efforts, as this results in the removal of morphological features often used for species identification. Therefore, a molecular-based, higher-throughput and cost-effective species identification method for fisheries management is required. High Resolution Melting (HRM) analysis is a relatively new and fast method, used after polymerase chain reaction (PCR) amplification for detecting nucleic acid sequence variation between species. In this study, PCR amplification of the 16S ribosomal RNA (*16S rRNA*) gene region was optimised. Thereafter, an HRM assay was validated as a species identification tool for houndshark species and further optimised for additional commercially exploited shark species, occurring in southern Africa. Overall, the HRM assay was optimised for thirteen species, but proven a functional species identification tool for seven of these species including some of the most commercially important species in South Africa: *Scylliogaleus quecketti*, *Mustelus mustelus*, *Mustelus mosis*, *Carcharhinus limbatus*, *Prionace glauca*, *Carcharhinus brachyurus* and *Carcharhinus obscurus*. For these seven species, based on appropriate HRM melt curve graphs, replicate sample identity could be verified consistently, with higher confidence percentage values. Further optimisation of the HRM assay is required as it was established that the assay was not suitable for case studies involving highly processed fins. Given the widespread exploitation of shark species and possible illegal trade of shark meat and fins, it is imperative to optimise all available identification tools, for better monitoring and enforcement of laws intended to protect these species. Altogether, the optimised HRM assay can be a valuable molecular tool for the preliminary screening of species, in situations where results in a short timeframe are critical.

3.1 Introduction

Sharks are a group of marine fish, many species of which are threatened by overexploitation to satisfy the high demand for internationally traded products, primarily shark fins (used in Asian soup dishes) and meat (Dent and Clarke 2015; Eriksson and Clarke 2015). In 2015, the Food and Agriculture Organisation of the United Nations (FAO) conservatively put the average declared value of total world shark fin imports at US $ 377.9 million per year, from 2000–2011 (Dent and Clarke 2015). Thus, shark fins are considered to be one of the most valuable marine products (Gallagher and Hammerschlag 2011). The protection for international trade of threatened shark species exists, under The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). CITES is the first line of defence against illegal wildlife trafficking and is a binding and multilateral environmental agreement between governments, with the aim to ensure that specimens of wild plants and animals are not threatened with their survival by international trade. To date, fourteen shark species, ten wedgefish species (*Rhinidae* spp.) and six giant guitarfish species (*Glaucostegus* spp.) are all CITES-listed in Appendix II (CITES 2019). For Appendix II CITES-listed species, exporting parties have the obligation to document throughout the supply chain that traded specimens were legally obtained, and that trade is not detrimental to the survival of the species (CITES 2017). In South Africa, in addition to complying to CITES regulations, the Department of Environmental Affairs (DEA) has a list of Threatened or Protected Species (TOPS). To date, there are fourteen elasmobranch species on the TOPS list. Firstly, the Critically Endangered species listed are all species within the family *Pristidae* (sawfishes), the Natal shyshark *Haploblepharus kistnasamyi* and whitespotted wedgefish *Rhynchobatus djiddensis*. Secondly, the Endangered species are scalloped hammerhead *Sphyrna lewini* and great hammerhead *Sphyrna mokarran*. Thirdly, the Vulnerable species are ragged-tooth shark *Carcharias taurus*, great white shark *Carcharadon carcharias*, whale shark *Rhincodon typus*, basking shark *Cetorhinus maximus* and flapnose houndshark *Scylliogaleus quecketti*. Lastly, the Protected species are tiger shark *Galeocerdo cuvier*, leopard catshark *Poroderma pantherinum*, striped catshark *Poroderma africanum* and sixgill sawshark *Pliotrema warreni* (DEA 2017). For all above-mentioned TOPS-listed species, certain

activities are restricted (for example selling, trading and buying) with a few exceptions for scientific, management, conservation or rehabilitation purposes (DEA 2017).

During port inspections, morphological identification of shark and ray species can be extremely difficult, as these animals are frequently processed at sea (Abercrombie *et al*. 2005; Akhilesh *et al*. 2014). During processing, specimens are "trunked" whereby the head, fins and guts are removed and subsequently, meristic and morphological criteria used for identification of specimens are removed (Smith and Benson 2001; da Silva and Bürgener 2007; Mendonça *et al*. 2010). For certain CITES-listed species, such as basking shark *C. maximus*, whale shark *R. typus* and great white shark *C. carcharias*, some products such as fins and carcasses can be readily identified by their large sizes or other morphological features (for example jaws and teeth for great whites) (FAO 2016). Silky shark *Carcharhinus falciformis*, scalloped hammerhead *Sphyrna lewini* and smooth hammerhead *Sphyrna zygaena* are the second, fourth and fifth most common shark species in fin trade (Cardeñosa *et al*. 2018a; Fields *et al*. 2018). For the aforementioned species, morphological characters can be used to identify most of the dried unprocessed fins (such as first dorsal and pectoral) and dressed carcasses (FAO 2016). A morphological species identification guide also exists for these, as well as other shark species – whereby the FAO developed a Shark Fin Guide document covering sixteen globally distributed shark species. The sixteen species are listed in **Table 3.1** and are either important due to their conservation status or because they are main targets for the international shark fin trade (FAO 2016). This Shark Fin Guide has relevance to fresh and partially dried first dorsal fins, whole caudal fins and pectoral fins for each of the sixteen species. The fact sheet in the guide contains measurements, diagnostic features and a photographic set of the dorsal, caudal and pectoral fins, also showing colour illustrations of the entire shark (FAO 2016).

Table 3.1: List of sixteen shark species covered by the Shark Fin Guide, for the identification of various shark fins (first dorsal, whole caudal and pectoral; fresh to partially dried), to the species-level (FAO 2016).

Scientific name	Common name	Scientific name	Common name
Rhincodon typus	whale	*Carcharhinus longimanus*	oceanic whitetip
Carcharias taurus	ragged-tooth	*Carcharhinus obscurus*	dusky
Alopias superciliosus	bigeye thresher	*Carcharhinus plumbeus*	sandbar
Carcharodon carcharias	great white	*Galeocerdo cuvier*	tiger
Isurus oxyrinchus	shortfin mako	*Prionace glauca*	blue
Isurus paucus	longfin mako	*Sphyrna lewini*	scalloped hammerhead
Lamna nasus	porbeagle	*Sphyrna mokarran*	great hammerhead
Carcharhinus falciformis	silky	*Sphyrna zygaena*	smooth hammerhead

The misidentification of sharks in fisheries operations is also a major concern in South African fisheries (da Silva *et al*. 2015), whereby target species include common smoothhound *Mustelus mustelus*, whitespotted smoothhound *Mustelus palumbes*, copper shark *Carcharhinus brachyurus*, tope shark *Galeorhinus galeus*, dusky shark *Carcharhinus obscurus* and broadnose sevengill *Notorynchus cepedianus* (da Silva and Bürgener 2007; da Silva *et al*. 2015). There has been longstanding confusion regarding the identification of various species within the genus *Mustelus* (López *et al*. 2006). Additionally, *Mustelus* spp. are often confused with other triakid species, including tope shark *G. galeus* and the non-commercialised spotted gully shark *Triakis megalopterus*. Species identification becomes paramount as *G. galeus* and *Mustelus* spp. are sometimes sold together in markets under the same local name (Farrell *et al*. 2009). Additionally, *T. megalopterus* is legislated as a non-commercial species, where it may not be marketed by commercial operators and is often mistaken for *M. mustelus* in fisheries (Booth *et al*. 2011). In an effort to collect accurate species-specific data from shark fisheries in South Africa, a dressed demersal identification key for identifying gutted and headed sharks was developed by da Silva (2007). The identification key is for commonly caught inshore species along the eastern and western Cape coasts of South Africa and includes measurements such as: black/white spots

present, number and size of dorsal fins, presence or absence of interdorsal ridge, a spine present on dorsal fin and two morphometric measurements (da Silva 2007). However, morphological identification approaches require analyses from expert taxonomists, are labour intensive and are not ideal when presented with a large number of specimens. Additionally, morphological identification becomes difficult when landed catches consist of shark fillets and fins (Smith and Benson 2001). Thus, molecular-based methods can assist with fisheries-related issues to identify and distinguish a large number of closely related shark species.

Molecular-based methods such as DNA barcoding, whereby the mitochondrial gene cytochrome *c* oxidase subunit I (*COI*) is used as a standardised molecular marker (Hebert *et al*. 2003), has been widely used in wildlife forensics (Dalton and Kotze 2011; Yan *et al*. 2013; Khedkar *et al*. 2014). Although this method is still continuously used, and successful, for the identification of shark species and shark products (Cardeñosa *et al*. 2017; Almerón-Souza *et al*. 2018; Feitosa *et al*. 2018), it requires post-PCR processes such as: agarose gel electrophoresis, PCR clean-up, sequencing reactions and sequence editing, alignment and analyses (Morgan *et al*. 2011). These post-PCR manipulations add cost and time to the processing of samples, as well as increasing the chances of contamination and human error (Rugman-Jones and Stouthamer 2016). For a large number of samples, a higher-throughput and relatively less expensive method such as High Resolution Melting (HRM) analysis is more suitable. High Resolution Melting analysis is a relatively new and fast post-PCR method, used for detecting nucleic acid sequence variation between species (Sharma *et al*. 2013). First, HRM analysis is a closed-tube assay that involves PCR amplification of the region of interest, in the presence of a DNA-binding dye that has a high fluorescence when bound to double-stranded DNA (dsDNA) but low fluorescence when unbound. This allows the quantitative monitoring of PCR amplification of the target region. Second, by increasing the temperature in small increments, the amplified target region is gradually denatured from dsDNA into single strands causing a decrease in fluorescence (as the dye is released), referred to as the "melt". Third, melt curves are produced whereby decreasing fluorescence is plotted against increasing temperature (Applied Biosystems 2010; Kapa Biosystems 2013). Each melt curve profile is characteristic of the amplicon, as melt curve temperatures typically vary with the size

and GC/AT ratio of the generated amplicon (Ririe *et al*. 1997). Importantly, HRM analysis has been successfully used to discriminate shark species within the blacktip complex (Morgan *et al*. 2011), as well as being used in the field for the detection of possible CITES-listed shark products, in order to detain shipments (Cardeñosa *et al*. 2018b). Previously, Maduna (2017) used an HRM assay based on a 16S ribosomal RNA (*16S rRNA*) gene region, to successfully distinguish six southern African houndshark species.

A more recent and robust shark species identification method is necessary, as enforcement and management of multispecies shark fisheries require that a large number of landed products are identified to the species-level. Furthermore, closely related species as well as unidentifiable shark products need to be identified in South African fisheries, in a more time and cost-effective manner. Thus, the aim of this chapter is to optimise a High Resolution Melting assay/method and apply this to commercially exploited and other vulnerable shark and ray species in South Africa. The methods include (1) optimising PCR amplification for the mitochondrial *16S rRNA* gene region, across all six southern African houndshark species and seven additional shark species and (2) validate and develop an HRM assay as a species identification tool for these southern African shark species. This chapter reports on how HRM analysis is able to detect and identify a range of commercially exploited shark species through species-specific melt curves, based on a *16S rRNA* gene region. Additionally, the advantages and disadvantages of the method, and the suitability thereof to case studies involving confiscated shark fins are highlighted.

3.2 Methodology

3.2.1 Optimisation of the 16S rRNA *PCR, using six southern African houndsharks and additional commercially exploited species*

At least two samples per selected species were used to test positive PCR amplification of the *16S rRNA* gene region. These samples consisted of six houndsharks as well as seven additional shark species (**Table 3.2**). Total genomic DNA (gDNA) was extracted from fin clip samples stored in 99 % ethanol using an adjusted cetyltrimethylammonium bromide (CTAB) DNA extraction protocol (Sambrook and Russell 2001). DNA quantification (ng/µl) and quality (absorbance ratio: 260/280 and 260/230) was determined for each sample using a NanoDrop Spectrophotometer; generally, for DNA a ratio of ~1.8 is acknowledged as "pure" (Technical Bulletin NanoDrop Spectrophotometers). Thereafter, DNA concentrations were adjusted accordingly to 50 ng/µl, and visualised on a 1.5 % (w/v) agarose electrophoresis gel, stained with ethidium bromide (EtBr), to determine DNA quality.

Table 3.2: List of six houndshark species and seven additional shark species, used for the High Resolution Melting assay positive control sample list. The PCR amplification of the *16S rRNA* region was performed using the Carch-16S-Uni primer pair from Maduna (2017).

Houndshark species		**Additional shark species**	
Scientific name	**Common name**	**Scientific name**	**Common name**
Scylliogaleus quecketti	flapnose houndshark	*Carcharhinus limbatus*	blacktip
Mustelus palumbes	whitespotted smoothhound	*Prionace glauca*	blue
Mustelus mustelus	common smoothhound	*Carcharhinus leucas*	bull
Galeorhinus galeus	tope	*Sphyrna zygaena*	smooth hammerhead
Mustelus mosis	arabian smoothhound	*Carcharhinus brachyurus*	copper
Triakis megalopterus	spotted gully	*Carcharhinus obscurus*	dusky
		Sphyrna lewini	scalloped hammerhead

A previous study by Maduna (2017) showed that the *16S rRNA* gene region was the most suitable for HRM analysis, out of an additional three gene regions tested: *12S-16S rRNA* gene region

(Iglésias *et al*. 2005) and nicotinamide adenine dehydrogenase subunits 2 and 4 (*ND2* and *ND4*; Boomer *et al*. 2012). A universal primer pair (**Table 3.3**) was designed from the conserved flanking regions of the *16S rRNA* gene region (~190 bp) (Maduna 2017).

Table 3.3: Universal primer pair used for PCR and the High Resolution Melting assay, to amplify the *16S rRNA* gene region of 190 bp.

Primer Name	Primer Sequence (5´–3´)	References
Carch-16S-UniF	AGAAGAGGTACAGCCCTTCTAA	Maduna 2017
Carch-16S-UniR	CCCAATAGGATAAAGGGGTTT	Maduna 2017

Initially, the PCR reaction and cycling conditions were carried out as recommended by Maduna (2017), but based on PCR results obtained, further optimisation was required to increase the specificity and sensitivity of the HRM assay. The following PCR reagents were tested: increasing the total reaction volume to 20 µl (from 10 µl), lowering the primer concentration (from 0.5 µM, to 0.4 µM, to 0.3 µM), lowering the primer concentration of one primer (to 0.3 µM) and increasing DNA concentration. The following PCR cycling conditions were tested: increased annealing temperature (from 57 °C to 60 °C), increased annealing time (from 30 s to 50 s), increased number of cycles (from 35 to 40), decreased elongation time (from 2 min to 30 s) and increased final extension temperature (from 60 °C to 72 °C). The optimised 20 µl PCR reaction included 1 X PCR buffer, 2.5 mM $MgCl_2$, 0.3 µM of each primer, 0.2 mM dNTPs, 0.5 U of Taq polymerase, 50 ng of DNA and Milli-Q water. The PCR reagents were adjusted by increasing the DNA concentration and decreasing the concentration of both Carch-16S-Uni primers. Amplification was done in the presence of negative (no template) controls and carried out in a SimpliAmp Thermal Cycler. The altered and final PCR cycling conditions used were as follows: an initial denaturation step at 95 °C for 2 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 50 s, elongation at 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The PCR amplicons, together with a negative control and a Promega 100 bp molecular size ladder, were visualised on a 2 % (w/v) agarose electrophoresis gel to determine the size of the gene fragment. The PCR amplicons (one amplicon per species) that amplified successfully were sequenced

using standard Sanger sequencing chemistry (BigDye® Terminator v.3.1 Cycle Sequencing Kit, Life Technologies, South Africa) and capillary electrophoresis performed at the DNA sequencing unit of Stellenbosch University – the Central Analytical Facility (CAF). Sequences were manually checked, edited and trimmed in MEGA7 (Kumar *et al*. 2016). Species-specific *16S rRNA* gene region sequences were confirmed by comparing sequences to the National Center for Biotechnology Information (NCBI) GenBank database (when species-specific sequences were available on the database).

3.2.2 Application of the HRM assay for species identification

Samples for the HRM assay consisted of one positive control sample for each species, as well as up to three replicates to determine Genotype Confidence Percentages (GCPs). A GCP is a value attributed to each species being compared to the genotype, with a value of 100 indicating an exact match. The number of replicates per species were dependent on sample availability and DNA quality.

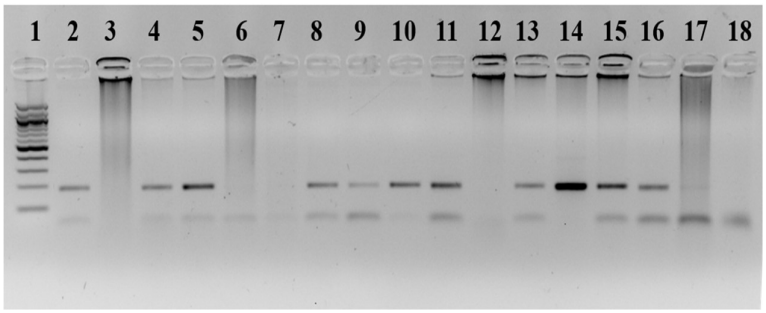
The HRM assay was conducted on a Rotor-Gene Q (Qiagen) and consisted of PCR amplification, DNA melting and end point fluorescence acquiring. For PCR amplification, the above-mentioned optimised PCR reagents and conditions were used, including the addition of SYTO® 9 green fluorescent nucleic acid stain at a final concentration of 5 µM. SYTO® 9 is a third generation DNA intercalating dye and is able to saturate all available sites within dsDNA, at high concentrations (Monis *et al*. 2005). It can be used to monitor the accumulation of PCR product as well as subsequent product melting. It has been reported that SYTO® 9 has better performance characteristics for real-time PCR and DNA melting curve analysis applications, compared to SYBR Green I (Monis *et al*. 2005). At the end of each 72 °C extension step of the PCR, fluorescence data were acquired on the HRM channel (excitation at 460 nm and detection at 510 nm). Following amplification, DNA melting was performed at temperatures ramping from 65 °C to 95 °C, rising by 0.1 °C increments every 2 s, and fluorescence data acquired on the HRM channel. Thereafter, melting curves were acquired on the HRM channel and visualised using the Rotor-Gene Q Software (v.2.3.1, Qiagen). A raw curve was plotted depicting the decreasing fluorescence against increasing temperature, showing two normalised temperature ranges (a leading and trailing range) which were consistent across all species per HRM run. Thereafter, a normalised raw curve was plotted, depicting the decreasing

fluorescence against increasing temperature. Melting temperature (Tm) was displayed by plotting the negative derivative of fluorescence (F) over temperature (T) (dF/dT) against temperature (°C). Difference graphs for each of the shark species were plotted as normalised fluorescence minus the normalised fluorescence of an individual species against temperature (°C). For species identification analyses, one sample per species was set as a reference species or "genotype" and the average HRM Genotype Confidence Percentages (GCPs) for the replicates for each species were estimated using the Rotor-Gene Q Software (v.2.3.1, Qiagen). In some cases, the replicate deviating the most was considered as an outlier and disregarded from further analyses. All samples and replicates used for the HRM assay were subjected to a 2 % (w/v) agarose electrophoresis gel, and methods followed as previously mentioned (in section 3.2.1). Additionally, if required for further analysis and validation, replicate samples were sequenced using standard Sanger sequencing chemistry (BigDye® Terminator v.3.1 Cycle Sequencing Kit, Life Technologies, South Africa) using the previously mentioned conditions (in section 3.2.1).

3.3 Results

3.3.1 Optimisation of the PCR and HRM assay for the 16S rRNA *gene region*

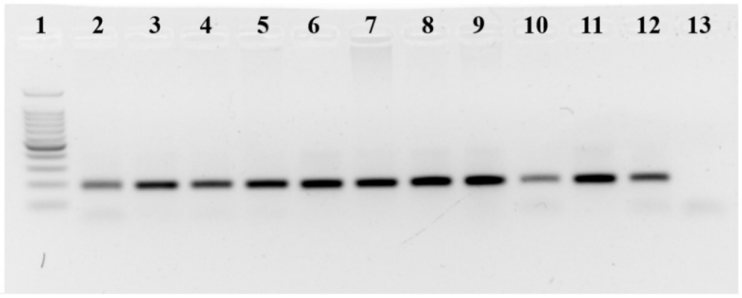
Successful amplification of the *16S rRNA* gene region (~190 bp) was obtained for the six houndshark species of Maduna (2017), as well as for seven additional shark species using recommended PCR reagents and cycling conditions (**Figure 3.1**). However, amplification was not consistent with low yield of PCR amplicons for certain samples and primer dimers were apparent.



Lane	Description
1	100 bp ladder
2 – 17	Samples 1 – 16
18	Negative control

Figure 3.1: 2 % (w/v) agarose gel electrophoresis image of *16S rRNA* PCR products (190 bp) before PCR optimisation, using the Carch-16S-Uni primer pair, using genomic DNA from houndshark and commercially exploited shark samples.

Polymerase chain reaction optimisation was therefore required, whereby a range of different concentrations of reagents and PCR cycling conditions were tested. The PCR cycling conditions were altered by increasing the number of cycles, increasing the annealing time, decreasing the elongation time and increasing the final elongation temperature. Results using the optimised conditions showed a decreased presence of primer dimers, an increased PCR product yield and more consistent amplification (**Figure 3.2**). Thus, these optimised PCR and cycling conditions were used for the amplification of the *16S rRNA* gene region during the HRM assay.



Lane	Description
1	100 bp ladder
2 – 12	Samples 1 – 11
13	Negative control

Figure 3.2: 2 % (w/v) agarose gel electrophoresis image of *16S rRNA* PCR products (190 bp) after PCR optimisation, using the Carch-16S-Uni primer pair, using genomic DNA from houndshark and commercially exploited shark samples.

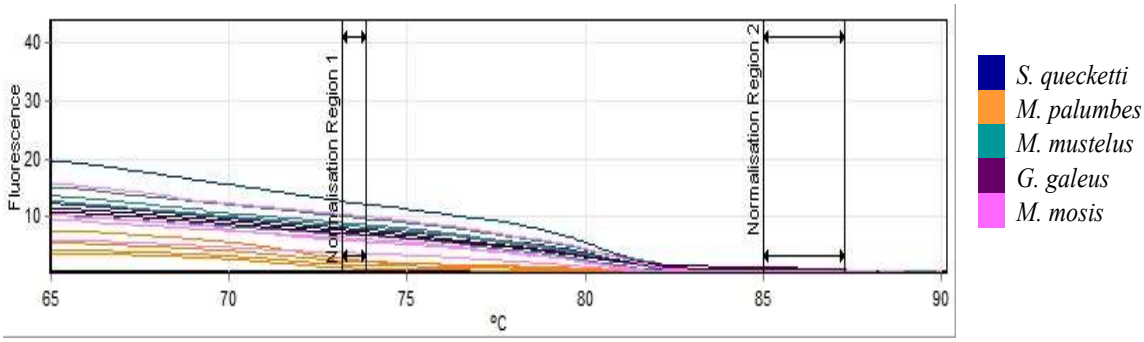
Successful sequences from the following species were compared to the *16S rRNA* sequences available on the NCBI GenBank database: tope shark *G. galeus*, common smoothhound *M. mustelus*, scalloped hammerhead *S. lewini*, smooth hammerhead *S. zygaena,* dusky shark *C. obscurus*, blue shark *P. glauca*, bull shark *C. leucas* and blacktip shark *C. limbatus*. Therefore, *16S rRNA* sequences were confirmed for these eight species. The remaining five sequences did not have publicly available *16S rRNA* sequences to be compared to; however, they were considered to be of the correct gene size, according to agarose electrophoresis gel results (~190 bp).

The HRM assay was further optimised by testing a range of SYTO® 9 dye concentrations (1.5 µM – 5 µM) and results based on fluorescence detection indicated an increased concentration of 5 µM SYTO® 9 resulted in the best fluorescence detection. A post-stain method whereby SYTO® 9 was added to sample tubes post-PCR was also tested; however, this was not ideal for fluorescence detection.

3.3.2 Results of the HRM assay for all selected commercially exploited species

For one of the houndshark species the spotted gully shark *Triakis megalopterus*, the replicate samples did not amplify, thus the positive genotype sample had no replicate samples to be compared to. Therefore, only five houndshark and seven additional exploited shark species were analysed for the HRM assay and raw data HRM graphs are shown in **Figure 3.3 a** and **Figure 3.3 b** respectively.

a



b

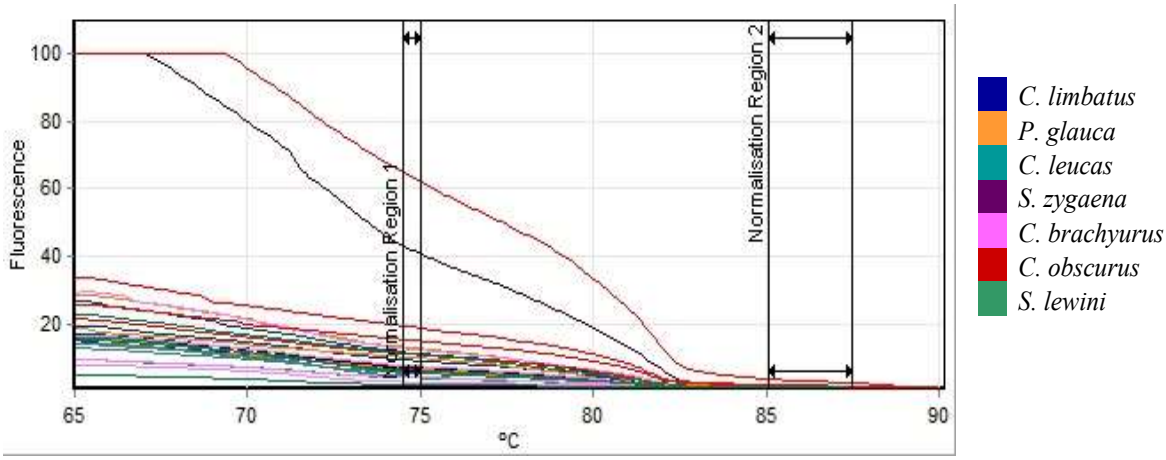
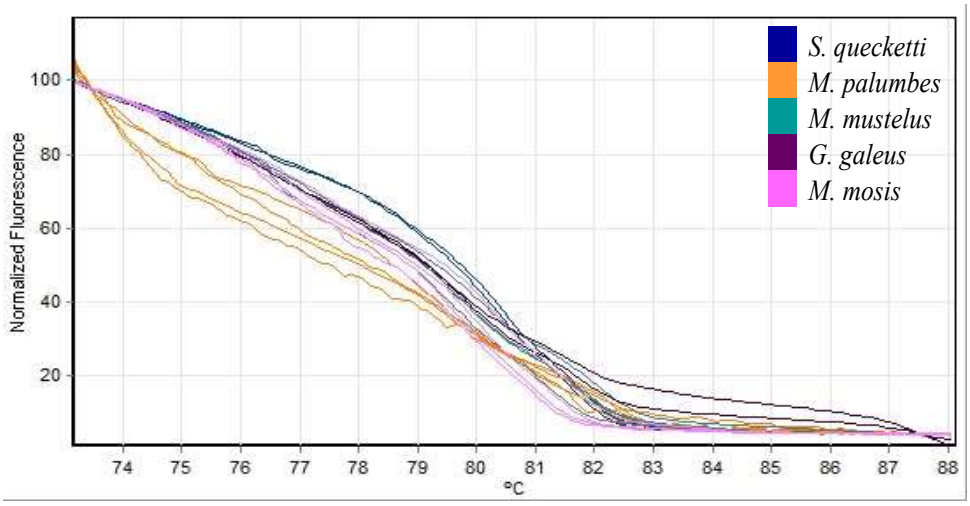


Figure 3.3: Raw High Resolution Melting (HRM) data for **(a)** five houndshark species and **(b)** seven additional shark species, indicating decreasing fluorescence vs. temperature (°C). The normalised regions per HRM assay run are also indicated.

The normalised fluorescence graphs for each HRM assay run are shown in **Figure 3.4**, based on normalisation of raw HRM assay data.

a



b

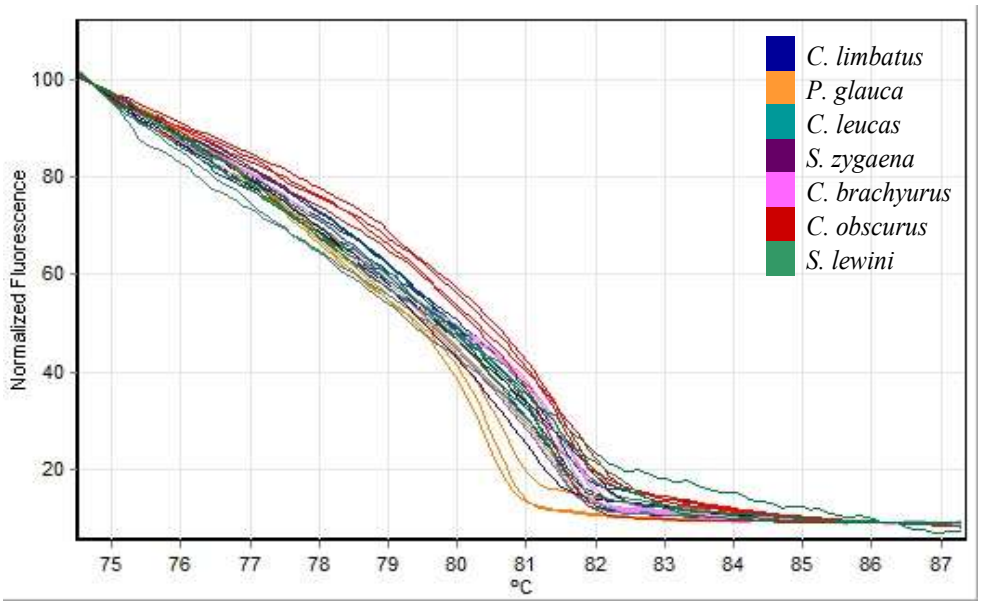


Figure 3.4: Normalised fluorescence graph for **(a)** five houndshark species and **(b)** seven additional shark species, indicating decreasing fluorescence vs. temperature (°C).

Melting temperature (Tm) was displayed by plotting the negative derivative of fluorescence (F) over temperature (T) (dF/dT) against temperature (°C), for the HRM assay of the five houndshark species (**Figure 3.5 a**) and the HRM assay for the seven additional shark species (**Figure 3.5 b**).

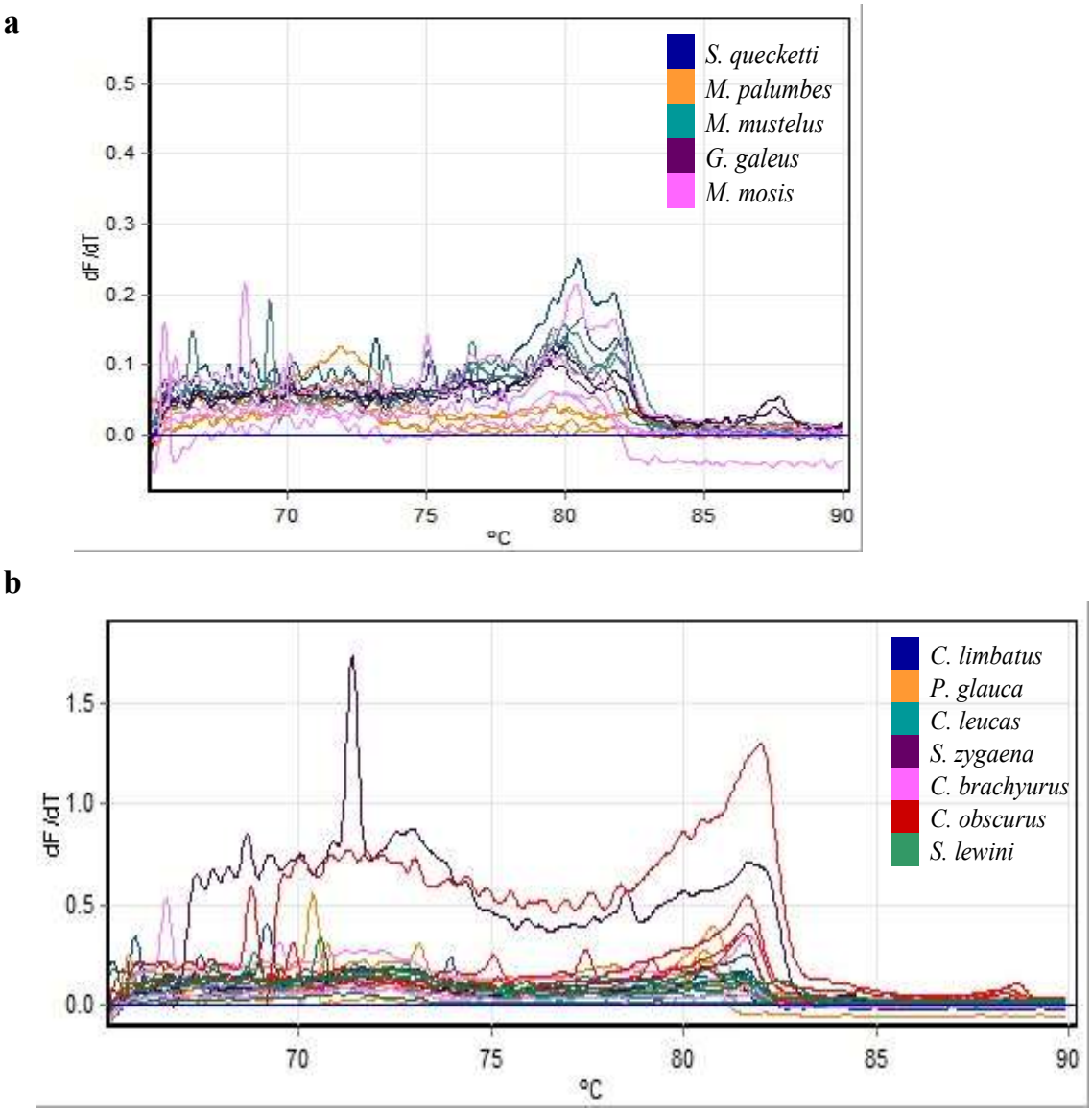


Figure 3.5: Melt curve profiles for **(a)** five houndshark species *Scylliogaleus quecketti, Mustelus palumbes, Mustelus mustelus, Galeorhinus galeus* and *Mustelus mosis*; and for **(b)** seven additional shark species *Carcharhinus limbatus, Prionace glauca, Carcharhinus leucas, Sphyrna zygaena, Carcharhinus brachyurus, Carcharhinus obscurus* and *Sphyrna lewini*. Melt curve profiles plotted as the negative derivative of fluorescence (F) over temperature (T) (dF/dT) vs. temperature (°C).

3.3.3 Success of the HRM assay for species identification of commercially exploited shark species

For each species, a genotype sample was selected and GCP values assigned to each replicate sample per species. A GCP is a value attributed to each sample being compared to the species reference genotype, with a value of 100 indicating an exact match. Based on GCP results for the five houndshark

species (**Table 3.4**) the following is noted: the replicate sample for *S. quecketti* was matched as the correct species with a high confidence value (87.46 %). *Mustelus palumbes* replicate samples show all three replicates matched to the correct species, with low confidence values (31.34 %, 0.45 % and 8.14 %). Two replicate samples for *M. mustelus* matched to the correct species with moderate confidence values (49.45 % and 50.36 %); however, one replicate sample was matched to *M. mosis* (47.02 %). It was later confirmed from sequencing results that the replicate sample was indeed *M. mustelus* and not *M. mosis*, and was therefore incorrectly identified by HRM analysis. The replicate sample for *G. galeus* was matched correctly with a moderate confidence value (36.68 %). Lastly, two replicate samples for *M. mosis* were matched correctly with confidence values of 30.85 % and 73.47 % respectively. However, one replicate sample was matched to *M. mustelus* (60.40 %) and later confirmed by sequencing results to in fact be *M. mosis*.

Table 3.4: Samples of five houndshark species, indicating the allocated genotype sample per species and Genotype Confidence Percentage (GCP) values assigned to each replicate sample.

Colour	Sample Name	Genotype	Genotype Confidence Percentage (GCP)
	S. que 1 +	*S. quecketti*	100
	S. que 2	*S. quecketti*	87.46
	M. pal 1 +	*M. palumbes*	100
	M. pal 2	*M. palumbes*	31.34
	M. pal 3	*M. palumbes*	0.45
	M. pal 4	*M. palumbes*	8.14
	M. mus 1 +	*M. mustelus*	100
	M. mus 2	*M. mustelus*	49.45
	M. mus 3	*M. mustelus*	50.36
	M. mus 4	*M. mosis*	47.02
	G. gal 1 +	*G. galeus*	100
	G. gal 2	*G. galeus*	36.68
	M. mos 1 +	*M. mosis*	100
	M. mos 2	*M. mosis*	30.85
	M. mos 3	*M. mosis*	73.47
	M. mos 4	*M. mustelus*	60.40

Based on GCP results for the seven additional shark species (**Table 3.5**), the following is noted: the replicate sample for *C. limbatus* was matched as the correct species with high confidence (91.56 %). Two replicate samples for *P. glauca* matched to the correct species, with high and moderate confidence values (84.93 % and 37.81 %). The replicate sample for *C. leucas* was matched correctly with a moderate confidence value (46.42 %). No replicate samples were matched correctly for *S. zygaena*, while sequence results confirm the replicate sample to be *S. zygaena*. Two replicate samples for *C. brachyurus* matched to the correct species, both with high confidence values (74.82 % and 76.05 %). The replicate samples for *C. obscurus* show that both matched to the correct species, with high and moderate confidence values (87.27 % and 51.97 %). Lastly, the replicate sample for *S. lewini* was matched correctly with a moderate confidence value (37.25 %).

Table 3.5: Seven additional shark species samples, indicating allocated genotype sample per species and Genotype Confidence Percentage (GCP) values assigned to each replicate sample.

Colour	Sample Name	Genotype	Genotype Confidence Percentage (GCP)
	C. lim 1 +	*C. limbatus*	100
	C. lim 2	*C. limbatus*	91.56
	P. gla 1 +	*P. glauca*	100
	P. gla 2	*P. glauca*	84.93
	P. gla 3	*P. glauca*	37.81
	C. leu 1 +	*C. leucas*	100
	C. leu 2	*C. leucas*	46.42
	S. zyg 1 +	*S. zygaena*	100
	S. zyg 2	*C. limbatus*	39.21
	C. bra 1 +	*C. brachyurus*	100
	C. bra 2	*C. brachyurus*	74.82
	C. bra 3	*C. brachyurus*	76.05
	C. obs 1 +	*C. obscurus*	100
	C. obs 2	*C. obscurus*	87.27
	C. obs 3	*C. obscurus*	51.97
	S. lew 1+	*S. lewini*	100
	S. lew 2	*S. lewini*	37.25

Difference graphs are displayed in Appendix A for each shark species assessed, whereby the normalised fluorescence minus the normalised fluorescence of an individual species is plotted against

temperature (°C). Six houndsharks and their replicates are shown as individual graphs with each species as the baseline, for: *S. quecketti*, *M. palumbes*, *M. mustelus*, *G. galeus*, *M. mosis* and *T. megalopterus* respectively (**Figure S3.1**–**Figure S3.6**). Seven other shark species and their replicates are shown as individual graphs with each species as the baseline, for: *C. limbatus*, *P. glauca*, *C. leucas*, *S. zygaena*, *C. brachyurus*, *C. obscurus* and *S. lewini* respectively (**Figure S3.7–Figure S3.13**). The significance of difference graphs is to better visualise small differences between melting curves of individual species in order to confirm genotypes and subsequently, species identification. Based on these graphs, the following is confirmed: *M. palumbes* is an outlier species among houndsharks, possibly due to poor amplification of all replicates (**Figure S3.1**–**Figure S3.6**), verified by low GCP values across all *M. palumbes* replicates (**Table 3.4**). For *M. mustelus* and *M. mosis*, one replicate of each is more similar to the alternate species (**Figure S3.3**–**Figure S3.5**), also apparent from GCP values (**Table 3.4**). Difference graphs for *C. limbatus*, *P. glauca*, *C. brachyurus* and *C. obscurus* show similar distinct curves across replicates within each species (**Figure S3.7–Figure S3.13**), also established from relatively higher GCP values across replicates (**Table 3.5**). Overall, distinct melt curves are observed for most species based on the difference graphs, confirming GCP values.

3.3.4 Success of HRM assay based on individual shark species

Based on HRM assay results for individual houndshark species: the samples for *S. quecketti* showed acceptable normalised fluorescence against temperature and dF/dT against temperature melt curve graphs (**Figure 3.6**). For the *16S rRNA* gene region of *S. quecketti*, the specific melt temperatures occurred at 81 °C and 83 °C (**Figure 3.6 b**). For the other four houndshark species, the melt temperatures were: 80 °C and 83 °C for *M. mustelus*, 80 °C and 81.5 °C for *G. galeus*, 81 °C and 83 °C for *M. mosis* and lastly, 80.5 °C and 83 °C for *T. megalopterus*. Poor amplification and subsequent fluorescence were observed for *M. palumbes* and thus specific melt temperatures could not be determined for samples of this species.

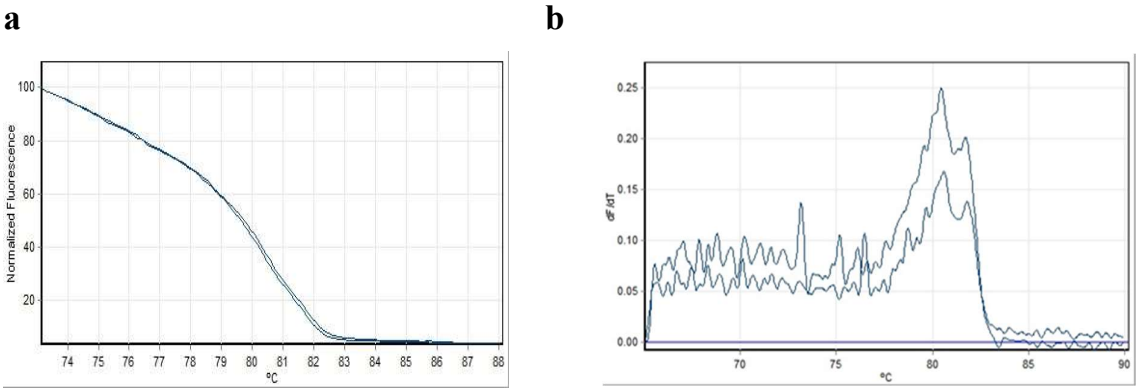


Figure 3.6: Samples of the flapnose houndshark *Scylliogaleus quecketti* shown as **(a)** normalised fluorescence graph, indicating decreasing fluorescence vs. temperature (°C) and **(b)** melting profile as a dF/dT vs. temperature (°C) graph.

Based on HRM assay results for the other individual shark species, acceptable normalised fluorescence against temperature and dF/dT against temperature melt curve graphs were observed for the samples for: *C. limbatus* (**Figure 3.7**), *P. glauca* (**Figure 3.8**), *C. brachyurus* (**Figure 3.9**) and *C. obscurus* (**Figure 3.10**). For the *16S rRNA* gene region, *C. limbatus* samples showed the melt temperature occurred at 81.5 °C (**Figure 3.7 b**), *P. glauca* samples at 80.5 °C (**Figure 3.8 b**), *C. brachyurus* samples at 82 °C (**Figure 3.9 b**) and *C. obscurus* samples at 82 °C (**Figure 3.10 b**). Poor amplification and subsequent fluorescence were observed for *C. leucas*, *S. zygaena* and *S. lewini*. Thus, specific melt temperatures for the *16S rRNA* gene region could not be determined for samples from these three species.

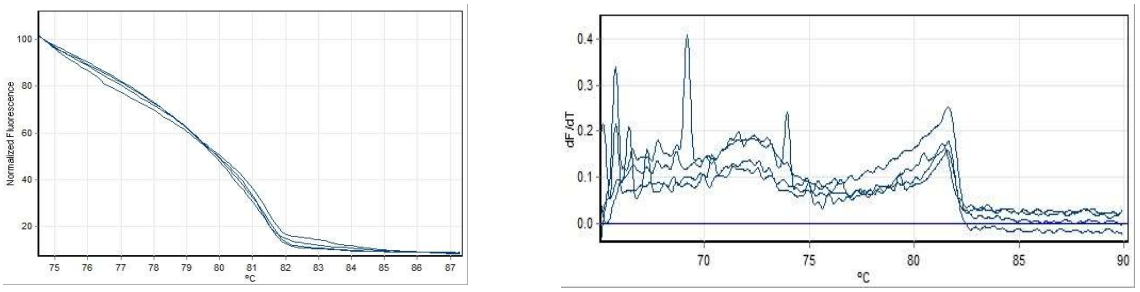


Figure 3.7: Samples of the blacktip shark *Carcharhinus limbatus* shown as **(a)** normalised fluorescence graph, indicating decreasing fluorescence vs. temperature (°C) and **(b)** melting profile as a dF/dT vs. temperature (°C) graph.

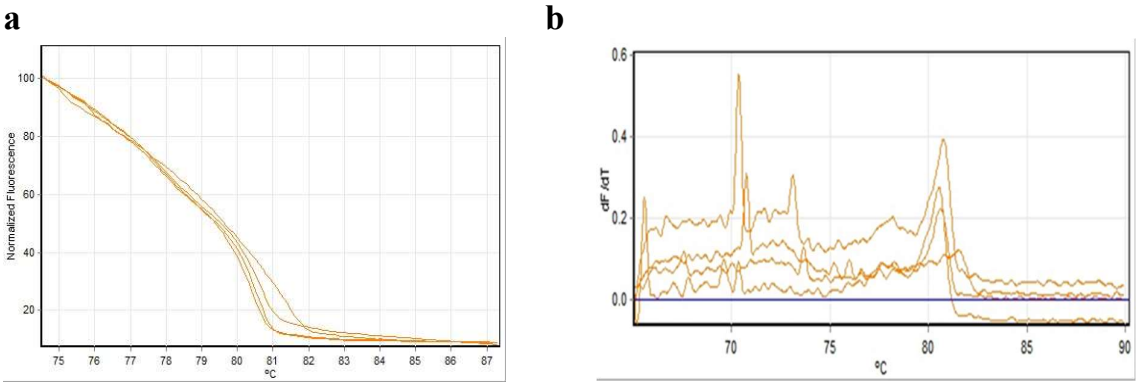


Figure 3.8: Samples of the blue shark *Prionace glauca* shown as **(a)** normalised fluorescence graph, indicating decreasing fluorescence vs. temperature (°C) and **(b)** melting profile as a dF/dT vs. temperature (°C) graph.

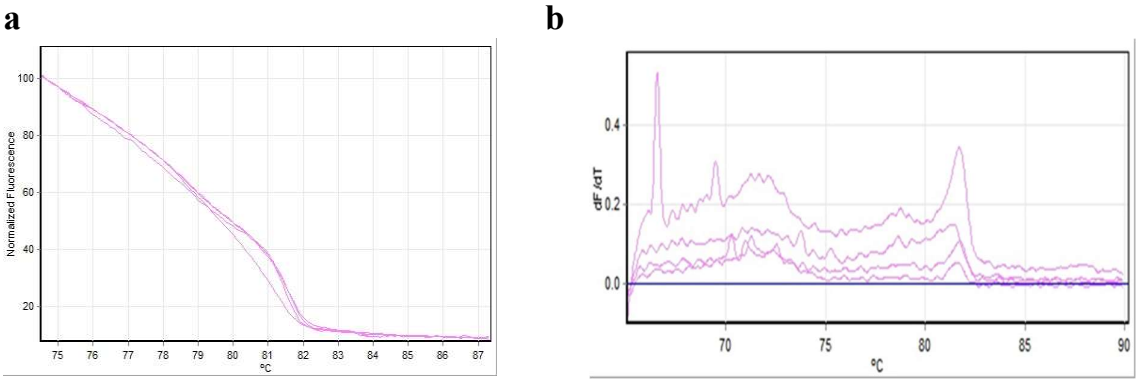


Figure 3.9: Samples of the copper shark *Carcharhinus brachyurus* shown as **(a)** normalised fluorescence graph, indicating decreasing fluorescence vs. temperature (°C) and **(b)** melting profile as a dF/dT vs. temperature (°C) graph.

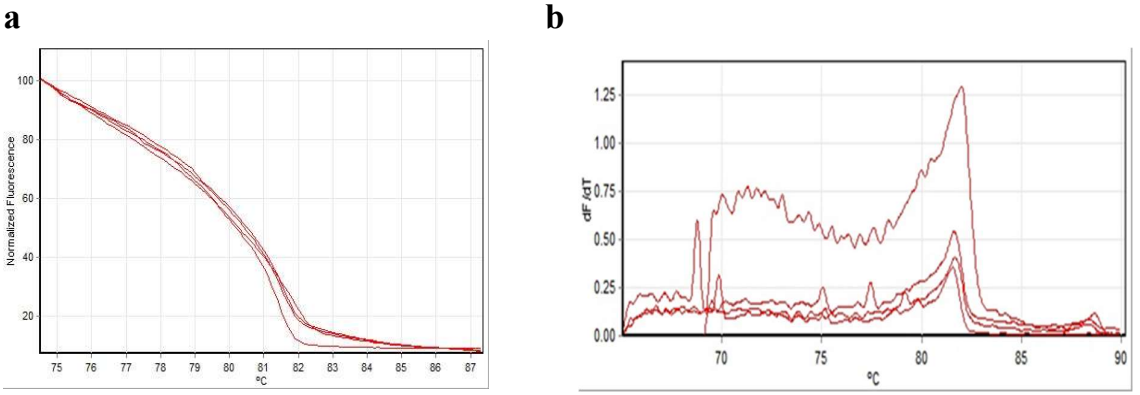


Figure 3.10: Samples of the dusky shark *Carcharhinus obscurus* shown as **(a)** normalised fluorescence graph, indicating decreasing fluorescence vs. temperature (°C) and **(b)** melting profile as a dF/dT vs. temperature (°C) graph.

3.4 Discussion

3.4.1 Success and suitability of the HRM assay for identifying commercially exploited shark species

In this study, PCR amplification of a *16S rRNA* gene region for HRM analysis was optimised to reduce primer dimers, increase PCR product yield and produce more consistent amplification across all selected shark species. An HRM assay was developed by Maduna (2017) for six houndshark species and thus further optimised based on these conditions. Species-specific HRM peaks/temperatures for the *16S rRNA* gene region could be validated *in silico* for five out of six houndshark species, and were determined for a further four out of seven additional shark species tested. More specifically, the five houndsharks are *Scylliogaleus quecketti*, *Mustelus mustelus*, *Galeorhinus galeus*, *Mustelus mosis* and *Triakis megalopterus* and four additional sharks are *Carcharhinus limbatus*, *Prionace glauca*, *Carcharhinus brachyurus* and *Carcharhinus obscurus*. Importantly, the individual melt curve graphs and melt peaks/temperatures for five houndshark species were directly comparable to HRM assay results of Maduna (2017), and thereby were validated in this study. Additionally, novel HRM melt curve graphs and melt peaks/temperatures based on the *16S rRNA* gene region were determined for four more shark species.

Based on replicate sample identity, GCP values indicate that eleven species in total were correctly identified. This included: *S. quecketti*, *M. palumbes*, *M. mustelus*, *G. galeus* and *M. mosis* as well as *C. limbatus, P. glauca, C. leucas, C. brachyurus, C. obscurus* and *S. lewini*. However, some replicate samples were called with relatively low (< 35 %) or moderate confidence (36–69 %) while not all replicate samples were called correctly, which was also reiterated from the difference graphs (in Appendix A). The low confidence values and inconsistencies could be attributed to sub-optimal PCR amplification, which was apparent on the HRM graphs. Poor amplification was evident from gel electrophoresis results for a few samples of *M. palumbes*, *G. galeus*, *T. megalopterus*, *C. leucas*, *S. zygaena* and *S. lewini*. Also, retrospectively, poor quantity and quality of DNA is predicted from NanoDrop ratios of some samples, which could have resulted in poor amplification and failure of the

HRM assay (Mukherjee *et al*. 2007). Successful HRM assay results requires PCR optimisation, as variable amplification may lead to an increased number of false positives (Millat *et al*. 2009). In this study, no false negatives were observed: all replicate samples were assigned to a particular species without variable genotypes. On the other hand, false positives were observed whereby some amplicons produced melt curves with subtle differences or showing decreased amplification and thus, were incorrectly called. This was also confirmed *via* Sanger sequencing (for example, the samples of *M. mustelus* and *M. mosis* observed in **Table 3.4**).

Overall, for seven commercially exploited shark species *S. quecketti*, *M. mustelus*, *M. mosis*, *C. limbatus, P. glauca*, *C. brachyurus* and *C. obscurus* – melt curve graphs were considered to be acceptable and resulted in mostly consistent species identification, with relatively higher confidence percentage values (> 70 %). Therefore, the HRM assay is considered to be useful as a species identification tool for seven shark species occurring in South Africa.

3.4.2 Further optimisation of the HRM assay

The abnormalities in fluorescence detection evident on the melt curve graphs suggest that further optimisation of the current HRM assay is necessary. These fluorescence abnormalities could be attributed to poor PCR amplification of individual samples, which is most likely a consequence of certain samples having a low DNA concentration and/or quality (Mukherjee *et al*. 2007). In future, especially for control samples, gDNA could be extracted freshly, as samples used for this study were ones readily available in the research group (Molecular Breeding and Biodiversity Group, Genetics Department, Stellenbosch University). The use of a Qiagen PCR master mix (more specific for the Rotor-Gene Q (Qiagen) machine) could also be considered with the addition of the fluorescent dye – to potentially improve reaction chemistry and consequently fluorescence detection. The use of a Qiagen-specific master mix can enable more specific amplification and reliable quantification for real-time PCR applications (Rotor-Gene Q – Pure Detection 2010). Optimisation of reaction and cycling conditions would not be required due to a more balanced combination of ions, which would minimise non-specific primer annealing (Rotor-Gene Q – Pure Detection 2010). An alternative

intercalating dye such as SYBR Green I could be tested, as it has also proven successful in HRM assay studies for the identification of shark species (Morgan *et al*. 2011; Cardeñosa *et al*. 2018b).

Furthermore, alternative gene regions could be used for successful HRM analysis. Previous studies report the success of the *ITS2* nuclear gene region (Cardeñosa *et al*. 2018b) as well as the mitochondrial *ND4* gene region (Morgan *et al*. 2011) utilised in an HRM assay, for species identification of sharks. Additionally, to overcome incorrect identification or false positive results (such as in the case of *M. mustelus* and *M. mosis*), species-specific primers could be used in a multiplex to potentially decrease error in HRM assay species identification (Mukherjee *et al*. 2007; Morgan *et al*. 2011; Cardeñosa *et al*. 2018b).

Therefore, although the developed HRM assay is proven successful for seven commercially important shark species, further optimisation of the HRM assay based on the *16S rRNA* gene region is necessary. This is already in progress by using freshly extracted DNA and possibly also a Qiagen master mix, an alternative intercalating fluorescent dye and/or gene region, and species-specific primers.

3.4.3 Application of the HRM assay and suitability for species identification in fisheries

Although not all samples across all species were called correctly, the HRM assay was able to identify seven commercially exploited species with relatively high confidence. These seven species (*S. quecketti*, *M. mustelus*, *M. mosis*, *C. limbatus, P. glauca*, *C. brachyurus* and *C. obscurus*) are all commonly caught in South African fisheries (da Silva 2007; da Silva and Bürgener 2007; da Silva *et al*. 2015). In terms of the houndsharks that were successfully identified using the HRM assay, the flapnose houndshark *Scylliogaleus quecketti*, is an endemic species to southern Africa and is on the TOPS list (DEA 2017). This species has a narrow geographic range and is classified on the IUCN Red List of Threatened Species as Vulnerable (Compagno 2009). *Scylliogaleus quecketti* occurs in shallow inshore areas and is targeted for its meat; however, little catch statistics from fisheries are available for this species (Compagno 2009; Ebert *et al*. 2013). The most commercially valuable demersal species, the common smoothhound *Mustelus mustelus*, is targeted for its white meat (da Silva 2007; da Silva and Bürgener 2007). This species is caught as both target and bycatch in fisheries, catch of *M. mustelus*

has been observed to be underreported and it is classified as Vulnerable (da Silva 2007; Serena *et al*. 2009). Little information is available regarding catch and trade data in southern Africa for the arabian smoothhound *Mustelus mosis* and this species is classified as Data Deficient (Valenti 2009). However, in India and Pakistan regions *M. mosis* is regularly fished and is reportedly sold for human consumption (Valenti 2009). Additionally, it was described as the second most abundant elasmobranch in Bahrain's fish markets (Moore and Peirce 2013). In terms of other species successfully identified using the HRM assay, the blacktip shark *Carcharhinus limbatus* and the blue shark *Prionace glauca* are both distributed worldwide in warm temperate, subtropical and tropical waters. They are also both classified as Near Threatened on the IUCN Red List of Threatened Species (Henderson *et al*. 2001; Burgess and Branstetter 2009). Worldwide, *C. limbatus* is targeted by commercial fisheries for its valued meat as well as fins for shark fin soup. In the USA, it is the second most important species landed commercially and its meat is considered superior to other shark species (Burgess and Branstetter 2009). In southern Africa, *C. limbatus* is processed and sold, among other shark species, under the name bronze whaler shark (also commonly known as copper shark) (da Silva and Bürgener 2007). *Prionace glauca* is reported to be the most widely distributed shark species (Last and Stevens 2009). In southern Africa, it is rarely targeted but caught as bycatch in vast numbers by pelagic longline fisheries (Petersen 2008). This species is a major component in the shark fin trade, whereby *P. glauca* was reported to comprise 17 % of fins in the Hong Kong fin trade (Clarke *et al*. 2006a; Clarke *et al*. 2006b). The copper shark *Carcharhinus brachyurus* is distributed in warm temperate and subtropical waters; however, populations are disjoint (Duffy and Gordon 2003). *Carcharhinus brachyurus* is classified as Near Threatened (Duffy and Gordon 2003) and in southern Africa this species is subjected to commercial fishing, sport fishing and is commonly caught in shark nets along beaches of KwaZulu-Natal (Compagno *et al*. 1989; Cliff and Dudley 2010). With regards to shark trade, *C. brachyurus* is one of the top five shark species in the South African demersal shark trade, due to its high flesh value when under 12 kg (da Silva and Bürgener 2007). Worldwide, this species is primarily caught for its meat and fins; however, fins from *C. brachyurus* make up a small percentage (less than 1 %) in major Hong Kong shark fin markets (Duffy and Gordon 2003; Fields *et al*. 2018). Lastly, the dusky shark

Carcharhinus obscurus is classified as Vulnerable and is a cosmopolitan species, with a patchy distribution in tropical and warm temperate seas (Last and Stevens 1994; Musick *et al*. 2009). Within South African waters, this species is targeted by longline fisheries, caught as bycatch by hake longline and trawl fisheries, and caught in shark nets along beaches of KwaZulu-Natal (Musick *et al*. 2009; DAFF 2013). Worldwide both meat and fins from *C. obscurus* are among the most valued in their respective categories (Musick *et al*. 2009). Specifically, their fins are highly popular due to their large size and high fin needle (ceratotrichia) content (Musick *et al*. 2009). Therefore, the commercial importance and/or worldwide trade of these sharks are highlighted, emphasising the importance of the HRM assay as a functional diagnostic tool for the identification of these seven exploited shark species.

In summary, several molecular methods for species identification exist in fisheries and have been successfully used for the identification of individual shark species. These methods include protein and gel electrophoresis identification methods (Smith and Benson 2001; Farrell *et al*. 2009); DNA barcoding and sequence-based identification methods (Ward *et al*. 2005; Blanco *et al*. 2008); as well as PCR multiplex methods (Farrell *et al*. 2009; Mendonça *et al*. 2010). However, these methods can require tedious post-PCR manipulations such as agarose gel electrophoresis, PCR clean-up, DNA sequencing and sequence editing, alignment and analyses (Morgan *et al*. 2011). High Resolution Melting analysis is advantageous due to the simplicity of the technique, as post-PCR processing of the samples is not required. After PCR amplification, melting curves are generated by fluorescence detection of a DNA-saturating dye (Applied Biosystems 2010; Kapa Biosystems 2013). High Resolution Melting analysis is a cost-effective post-PCR technique and can allow for successful species identification at a higher-throughput level (Millat *et al*. 2009). The HRM assay optimised in this study has the potential to be applied as a useful species diagnostic tool, for the relevant shark species. Compared to a DNA amplification and sequencing-based species identification approach, the HRM assay is more cost-effective, less laborious and less time-consuming (Millat *et al*. 2009). High Resolution Melting analysis has previously been used for species identification, whereby it was successfully used for discriminating between three closely related whaler shark species (*C. tilstoni*, *C. limbatus* and *C. amblyrhynchoides*) in Australian waters (Morgan *et al*. 2011). Additionally, the

optimised HRM assay has potential for monitoring trade in shark products as an initial step in order to detain a shipment (Cardeñosa *et al*. 2018b), or allow for additional time upon initial identification. However, it is important to note that the assay would not be ideal for case studies involving samples with degraded or poor-quality DNA, as HRM assays are highly sensitive and specific (Millat *et al*. 2009). Additionally, subsequent sequencing is still recommendable for validation and confirmation of the species identity. Therefore, the HRM assay developed in the current study could be used as an initial step for species identification of seven commercially exploited species, when species information is required in a short timeframe.

3.5 Conclusion

In summary, this chapter reports on the validation of an HRM assay for five houndshark species, as well as the optimisation of this assay for an additional four southern African exploited shark species. The potential use of this HRM assay, based on a *16S rRNA* gene region, is assessed as a species identification tool for fisheries management purposes. The importance of the HRM assay as a functional diagnostic tool for the identification of at least seven southern African exploited shark species is highlighted, based on the commercial importance and/or worldwide trade of these species. Three of the most commercially important shark species found regionally and a southern African endemic houndshark are included. Further optimisation of the HRM assay is required and it should be used in combination with additional molecular species identification techniques. Nonetheless, in future, this HRM assay could be used for the inspection of detained consignments for example, when species identification information is required in a relatively short timeframe.

CHAPTER 4

Concluding remarks and future research

4.1 Introduction

Worldwide, ocean and coastal threat is driven by the rapid expansion of fisheries and global trade markets, whereby overfishing activities and habitat degradation have severely altered shark and ray populations (Stevens *et al*. 2000; Dudley and Simpfendorfer 2006; Ferretti *et al*. 2010). A main driver of shark fishing is to meet the demand for fins (not only from sharks, but also sawfishes and wedgefishes) which are exported from various countries around the world to Hong Kong (Dulvy *et al*. 2014). These threats have placed shark species at a high risk of becoming endangered or extinct (Myers and Worm 2003). Numerous studies report a widespread decline of chondrichthyan (sharks, skates, rays and chimaeras) populations (Dulvy *et al*. 2014; Davidson *et al*. 2016; Espinoza *et al*. 2018), and in many regions chondrichthyan populations have declined to unsustainable levels (Ward-Paige *et al*. 2010; Davidson *et al*. 2016; Spaet *et al*. 2016). Consequently, chondrichthyans are facing possibly the largest crisis of their 420 million year existence (Simpfendorfer and Dulvy 2017).

Over 35 % of marine species occurring in southern Africa are endemic species (Branch *et al*. 2016) and this region is highlighted as one of the seven hotspots under coastal threat in the world (Dulvy *et al*. 2014). Accordingly, the southern African coastline has been identified as a conservation priority (Dulvy *et al*. 2014; Davidson and Dulvy 2017; Stein *et al*. 2018). Reportedly, 100 out of 204 chondrichthyan species that occur in southern Africa are impacted by fisheries and are caught in significant quantities by non-directed and directed shark fisheries (da Silva *et al*. 2015, 2018). Additionally, the misidentification of shark species in fisheries operations is a major concern (da Silva *et al*. 2015). Accurate species identification is crucial for: the monitoring of fisheries' catches (both harvests and discards), defining the species composition of a fishery, and assessing fishery stocks (Tillet *et al*. 2012; Bester-van der Merwe and Gledhill 2015). Sharks are primarily processed at sea, whereby morphological features such as heads and fins of specimens are removed, making species identification

almost impossible (Smith and Benson 2001). Therefore, the use of molecular identification methods has increased: to identify species composition of fisheries (Sembiring *et al*. 2015; Bineesh *et al*. 2017); to identify threatened shark species being traded (Feitosa *et al*. 2018; Hobbs *et al*. 2019); to perform species identification of shark fins and products in trade (Steinke *et al*. 2017; Abercrombie *et al*. 2018; Hobbs *et al*. 2019); and to report on seafood fraud or mislabelling (Barbuto *et al*. 2010; Cawthorn *et al*. 2012).

The frequent occurrence of species misidentification and the fact that specimens are often landed in large numbers by fisheries prevents conservation and management plans (Abercrombie *et al*. 2005; Bester-van der Merwe and Gledhill 2015; Smart *et al*. 2016). Therefore, the research presented in this thesis aimed at developing molecular identification assays applicable to southern African fisheries case studies, with focus on exploited elasmobranch species, for trade monitoring and compliance in fisheries operating in this region.

4.2 Summary and discussion of research findings

In South Africa's diverse fishery sectors, almost 50 % of chondrichthyan species occurring in southern Africa are regularly targeted or taken as bycatch (da Silva *et al*. 2015, 2018). Additionally, catches are often underreported and the misidentification and/or grouping of species is common (da Silva *et al*. 2015). An increase of molecular-based studies on elasmobranchs has been observed due to the urgent need to address critical conservation issues (Dudgeon *et al*. 2012), especially studies employing molecular methods as tools for species identification. Molecular-based approaches are used for the identification of shark fins; however, fins are frequently in a processed state containing degraded DNA and often incompatible with the use of traditional identification methods (Abercrombie *et al*. 2018). Additionally, seafood fraud in South Africa is a reality and a concern for seafood consumers (Cawthorn *et al*. 2015). Accordingly, Chapter 2 investigated the use of a standard DNA barcoding method, as well as an optimised mini-barcoding multiplex assay (based on a *COI* gene region) applied to marine forensic case studies; mostly involving confiscated shark fins from southern Africa.

The seafood case study, using a traditional barcoding method, showed that not all fish products' main ingredients are from South Africa and ambiguous labelling appears to be common. The findings in Chapter 2 were congruent with previous studies, and showed that misrepresentation at the species-level as well as at the country-of-origin level does occur within South African seafood products (Cawthorn *et al*. 2011), confirming the ongoing misrepresentation of seafood labelling. These factors increase the possibility of consuming fish or possibly shark species unknowingly, and thus increase the risk of unsafe mercury intake, fish allergy and allergic reactions. Furthermore, the limitations of only using the traditional Fish1 *COI* barcoding primers for shark forensic species identification purposes are highlighted in Chapter 2. When using traditional methods, only a 22 % species identification success rate was obtained; whereas the mini-barcoding assay showed a 100 % success rate of the same samples. The mini-barcoding assay was optimised based on commercially exploited elasmobranch species and applied to a variety of southern African forensic case studies involving confiscated shark fins. The findings indicated that larger pelagic shark species are the main species targeted due to their more sought-after and larger fin size (*Carcharhinus* spp.). Of most concern, is that shark fins from CITES-listed and endangered species (*Sphyrna lewini*, *Isurus oxyrinchus* and *Rhynchobatus djiddensis*) were identified in one of the case studies. Fins from these species were possibly harvested for illegal trade. Recently, a study on the species composition of the shark fin trade, based on markets in Hong Kong, indicated that fins from CITES-listed as well as endangered species still exist in significant proportions (Cardeñosa *et al*. 2018a; Fields *et al*. 2018). From the case studies, it is also evident that South Africa acts as a possible intermediate transportation zone (between other Western Indian Ocean countries and Hong Kong) for the exportation of shark fins. Overall, the mini-barcoding assay was optimised successfully and proven to be ideal for species identification of shark fin samples and possibly other shark products (such as salted shark meat). Noteworthy is that some of the samples that were successfully identified were in a processed condition and possibly only contained DNA in a degraded state. This is the first study to report on the optimisation of a mini-barcoding assay for case studies involving confiscated shark fins in the South Africa area; in order to record the possible illegal trade of CITES-listed and endangered shark species from this region.

Molecular-based tools for wildlife forensics such as DNA barcoding (Ward *et al*. 2005; Naylor *et al*. 2012; Hellberg *et al*. 2019) and species-specific polymerase chain reaction (PCR) assays (Abercrombie *et al*. 2005; Cardeñosa *et al*. 2017) are the most reliable species identification approaches available to detect illicit shark fin and product trade (Abercrombie *et al*. 2018; Fields *et al*. 2019). However, shark fins and products are sometimes visually indistinguishable and frequently traded in high volumes. Shipments often have to be screened in a short timeframe, and together with capacity and cost factors, the use of DNA barcoding is limited (Cardeñosa *et al*. 2018b). High Resolution Melting (HRM) analysis is a closed-tube and post-PCR technique, allowing for successful species identification at a higher-throughput level (Millat *et al*. 2009), making it a more rapid and cost-effective approach for bigger consignments.

A new and robust shark species identification method is necessary, as enforcement and management of multispecies shark fisheries require that a large number of landed products are identified to the species-level. Furthermore, closely related species, as well as unidentifiable shark products, need to be identified in South African fisheries in a more time and cost-effective manner. Accordingly, Chapter 3 investigated the use of an HRM assay/method optimised for the successful identification of commercially exploited elasmobranch species; ultimately to be applied to assist fisheries management in southern Africa.

Maduna (2017) reported that an HRM assay, based on a *16S rRNA* gene region, was successful for the identification of six southern African houndshark species. In Chapter 3, the HRM assay was further optimised and validated for five of the houndsharks: *Scylliogaleus quecketti*, *Mustelus mustelus*, *Galeorhinus galeus*, *Mustelus mosis* and *Triakis megalopterus*. Additionally, this study is the first to report on how the optimised HRM assay is able to successfully identify another four shark species: *Carcharhinus limbatus*, *Prionace glauca*, *Carcharhinus brachyurus* and *Carcharhinus obscurus*. Established from the above, species-specific HRM profiles could be validated for five houndsharks and determined for another four shark species. Based on replicate sample identity, Genotype Confidence Percentage (GCP) values indicate that eleven species in total were correctly identified. This included five houndsharks *S. quecketti*, *M. palumbes*, *M. mustelus*, *G. galeus* and *M. mosis* as well as six

additional sharks *C. limbatus, P. glauca, C. leucas, C. brachyurus, C. obscurus* and *S. lewini*. However, some replicate samples were called with relatively low confidence (< 35 %) and not all replicate samples were called correctly, therefore inconsistent species identification was apparent. Combining results from species-specific HRM profiles and GCP values of replicate samples per species, the HRM assay optimised here has the potential to be a functional species diagnostic tool for seven commercially exploited shark species in total. These shark species are: flapnose houndshark *S. quecketti*, common smoothhound *M. mustelus*, arabian smoothhound *M. mosis*, blacktip shark *C. limbatus*, blue shark *P. glauca*, copper shark *C. brachyurus* and dusky shark *C. obscurus*. These species are all commercially important (including the most commercially important shark *M. mustelus*) and are traded worldwide for their fins and/or products. When species identification information is required for a large number of samples in a timely manner, an HRM assay can be considered as a higher-throughput, cost-effective, less laborious and less time-consuming approach (Millat *et al*. 2009). The optimised HRM assay presented in this study could be used as an initial step for species identification, especially when species identification information is required in a short timeframe, but ideally should be used in combination with other molecular species identification techniques for validation.

In summary, the molecular assays developed and optimised for species identification presented in this thesis are applicable to southern African fisheries case studies, with focus on exploited elasmobranch species, for trade monitoring and compliance in fisheries operating in this region.

4.3 Project limitations and future prospects

Although not exhaustive, the research presented in this thesis provided valuable insights into the contemporary species composition of elasmobranch species involved in trade in the southern African region. However, it is noted that this study provides a small-scale assessment based on sample size limitations. An increase in sample size or increase of case studies could provide a more comprehensive portrayal of elasmobranch exploitation, as well as the shark fin and product trade, for the region. Limitations for the molecular identification assays developed here are also noted. For the mini-barcoding multiplex assay, successful species identification is not always possible for closely

related species (such as *Mustelus* spp. and some *Carcharhinus* spp.) due to shorter *COI* gene regions used (150 bp and 200 bp regions) and thus, these results suggest that multiple *COI* gene fragments should be assessed. For HRM analysis, which is an identification tool based on presence or absence, traditional sequencing-based methods following the assay are still required (when confidence levels are low) for the validation of species identification results. Although it was proven as a functional identification tool, this was only for seven commercially exploited shark species and therefore a limited range of identification. Future work should include further optimisation of the HRM assay, in order to increase the consistency of fluorescence detection and thus identification, and also include optimisation for more species, especially the CITES-listed species occurring in the region.

The molecular identification assays presented in this study are applicable and optimised for the identification of elasmobranch fins, meat and products. Therefore, a procedure is presented for future case studies, outlining how each assay is useful under specific conditions. The first step for identification of specimens is morphological-based identification, using morphological features (such as fins, scales and heads). Shark species identification guides can be utilised; however, this could require expert taxonomists and could be a time-consuming process. A Shark Fin Guide covering sixteen globally distributed shark species is available and includes CITES-listed shark species, as well as shark species directly targeted for their fins (FAO 2016). Furthermore, an identification key for identifying gutted and headed sharks is available for morphological identification of South African demersal species (da Silva 2007). If identification cannot be determined morphologically or if uncertainty exists, molecular-based identification assays should be employed. The choice of the molecular assay for species identification would depend on a number of factors including the state of the samples (fresh, semi-processed or processed) and the time available. For unprocessed shark products, such as wet or dried fins and meat fillets with the skin still attached, the traditional barcoding method using the 652 bp *COI* gene region (amplified using the Fish1 primer pair from Ward *et al*. (2005)) will suffice; as outlined in Chapter 2. For processed shark products, such as dried and chemically treated fins/products with skin removed (and are a yellow or golden colour), the optimised mini-barcoding multiplex assay will be more appropriate; also as outlined in Chapter 2. Processed samples will most likely contain degraded

DNA and are incompatible with the use of traditional identification methods (Abercrombie *et al*. 2018). The mini-barcoding multiplex assay allows for shorter *COI* gene regions (150 bp and 200 bp) to be amplified (Cardeñosa *et al*. 2017) and is more compatible for degraded or fragmented DNA. Lastly, if species identification information is required in a short timeframe or to detain a shipment, the optimised HRM assay could be used as a preliminary screening tool to identify the presence or absence of seven commercially exploited species; full methodology as outlined in Chapter 3. For the HRM method to be successful; however, specimens should not be in a processed state while subsequent sequencing-based methodology may still be necessary to confirm results.

4.4 Final remarks

In conclusion, the research presented in this thesis underlines a practical approach in order to determine which molecular identification assay is most applicable when presented with elasmobranch specimens, in various conditions or states of processing. The molecular assays presented in this study were successfully optimised for the identification of southern African commercially exploited elasmobranch species. This is the first study to report on possible illegal trade of CITES-listed and endangered shark species from the southern African region, through the use of an optimised mini-barcoding assay. Additionally, it is the first study to optimise an HRM assay based on a *16S rRNA* gene region for the identification of seven commercially exploited sharks, including the most commercially important and an endemic houndshark species. Ultimately, the importance for molecular-based species identification is highlighted, especially when presented with processed, degraded or disguised specimens. Given the widespread exploitation of elasmobranch species and possible illegal trade of shark fins and products, it is imperative to optimise all available species identification tools, for better monitoring and enforcement of the illicit elasmobranch trade.

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Appendix A:

Supplementary material for Chapter 3

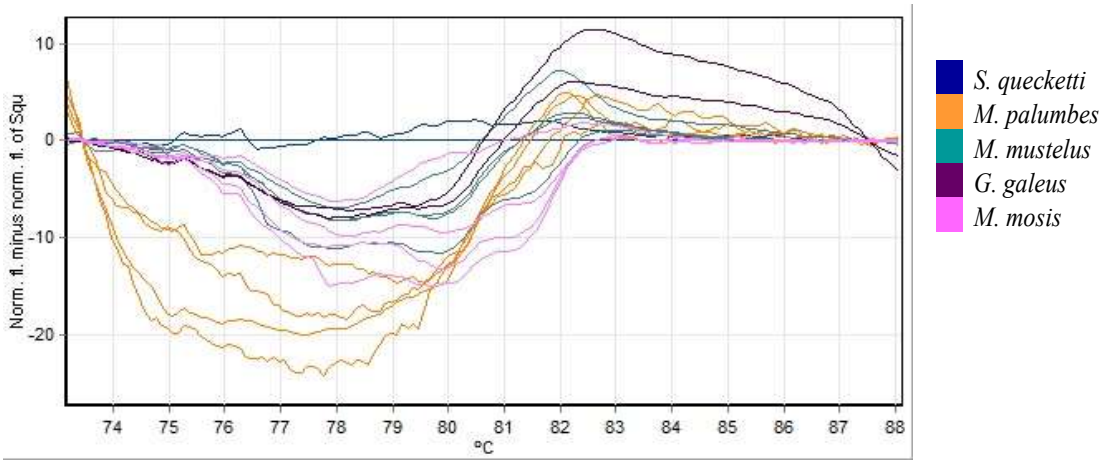

Figure S3.1: Difference plot for all houndshark replicates, using *Scylliogaleus quecketti* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Scylliogaleus quecketti* samples vs. temperature (°C).

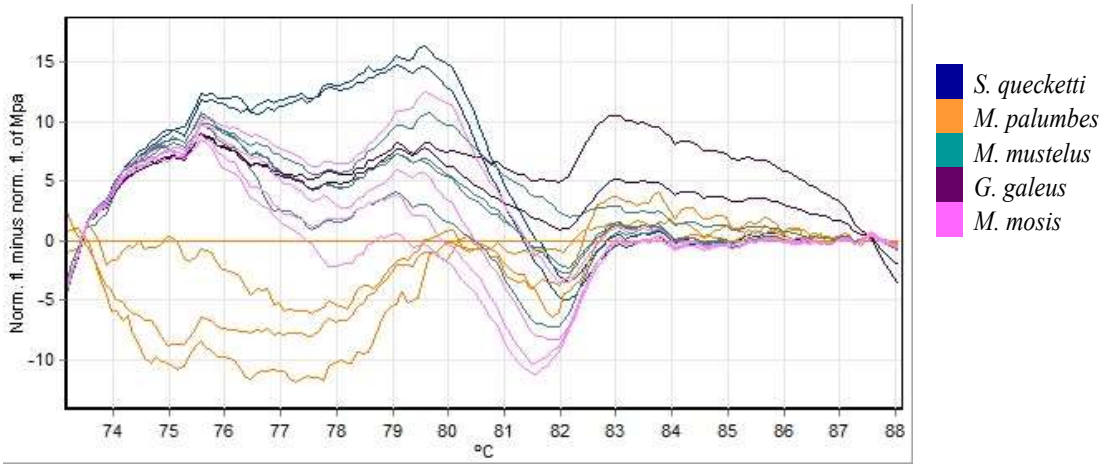

Figure S3.2: Difference plot for all houndshark replicates, using *Mustelus palumbes* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Mustelus palumbes* samples vs. temperature (°C).

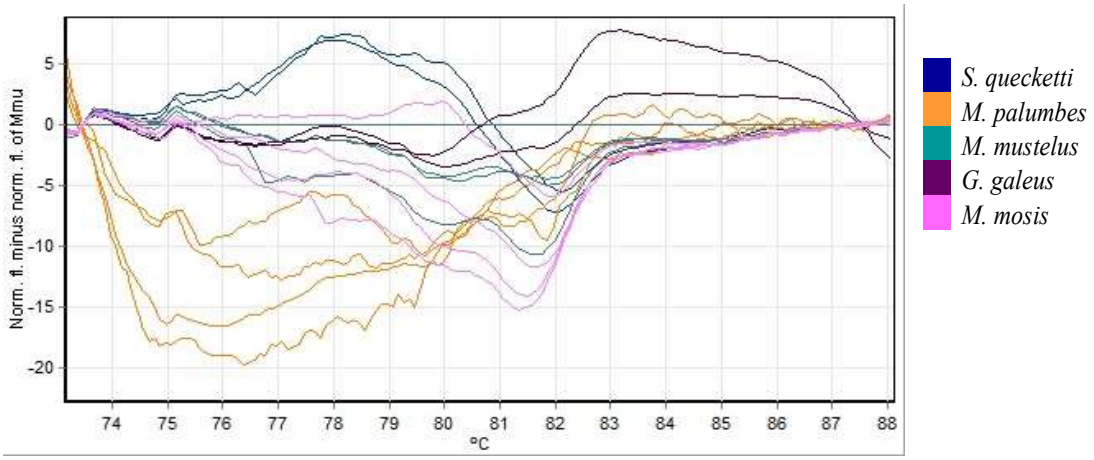

Figure S3.3: Difference plot for all houndshark replicates, using *Mustelus mustelus* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Mustelus mustelus* samples vs. temperature (°C).

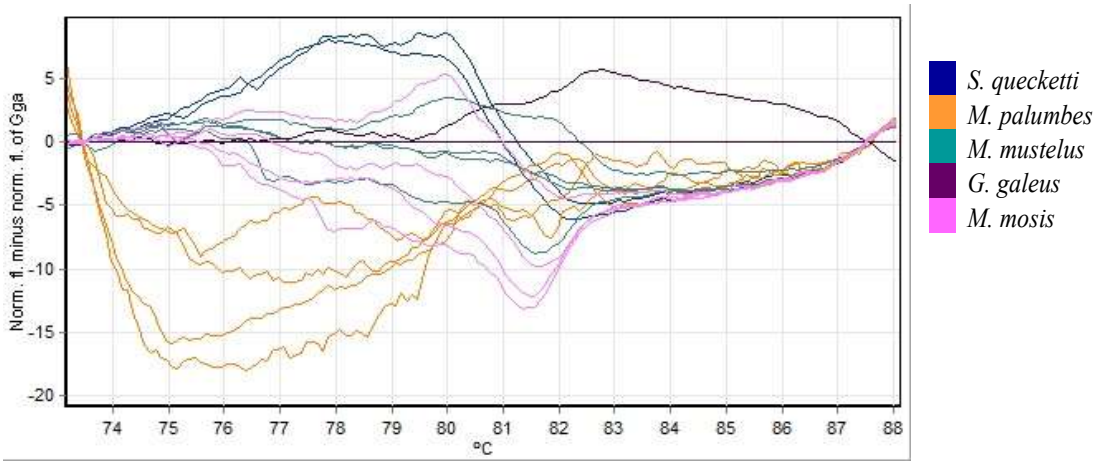

Figure S3.4: Difference plot for all houndshark replicates, using *Galeorhinus galeus* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Galeorhinus galeus* samples vs. temperature (°C).

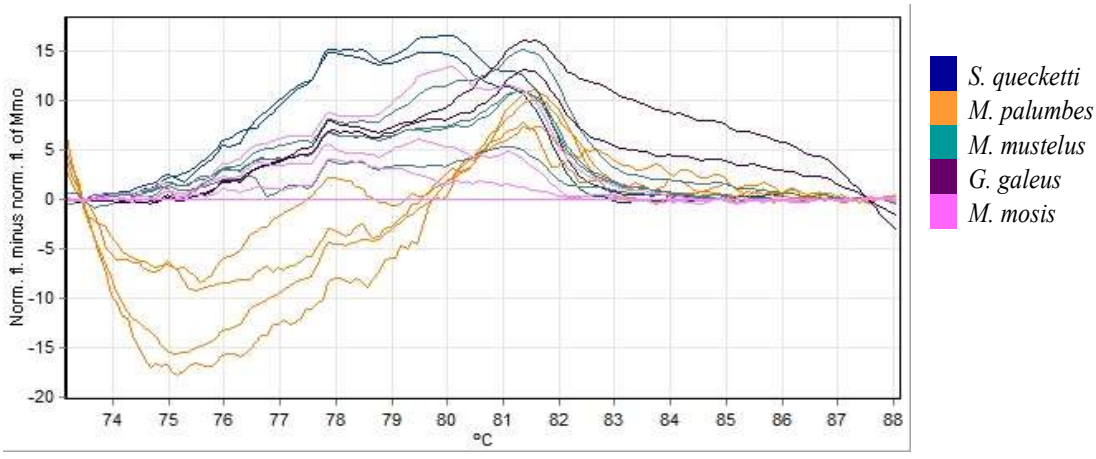

Figure S3.5: Difference plot for all houndshark replicates, using *Mustelus mosis* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Mustelus mosis* samples vs. temperature (°C).

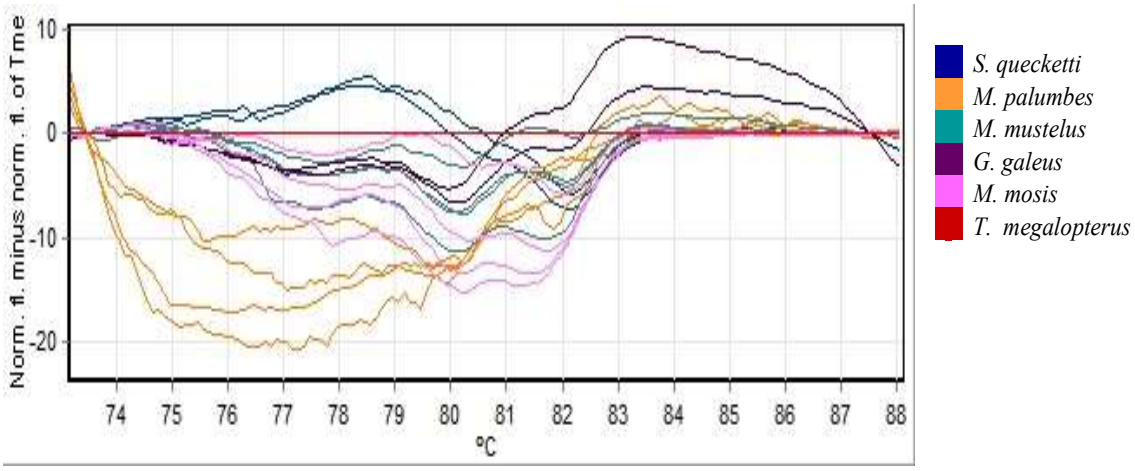

Figure S3.6: Difference plot for all houndshark replicates, using *Triakis megalopterus* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of one *Triakis megalopterus* sample vs. temperature (°C).

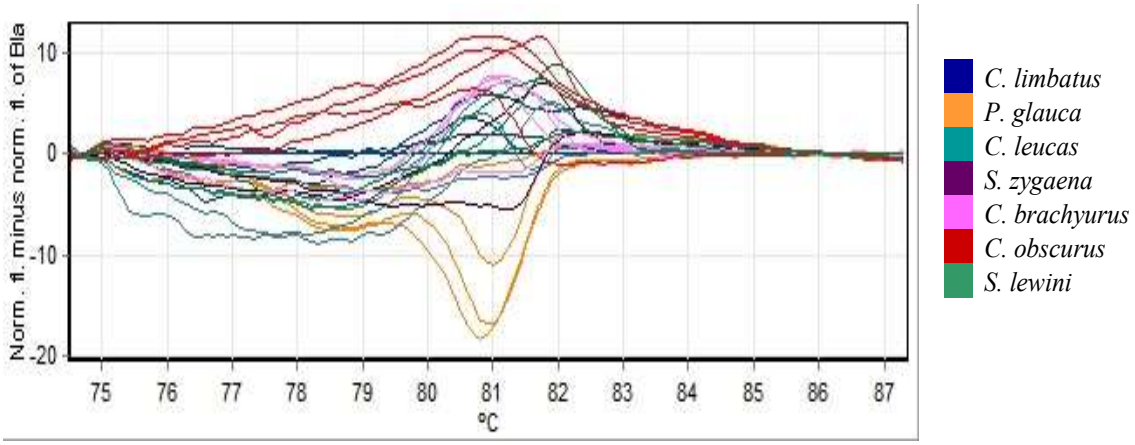

Figure S3.7: Difference plot for all additional shark replicate samples, using *Carcharhinus limbatus* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Carcharhinus limbatus* samples vs. temperature (°C).

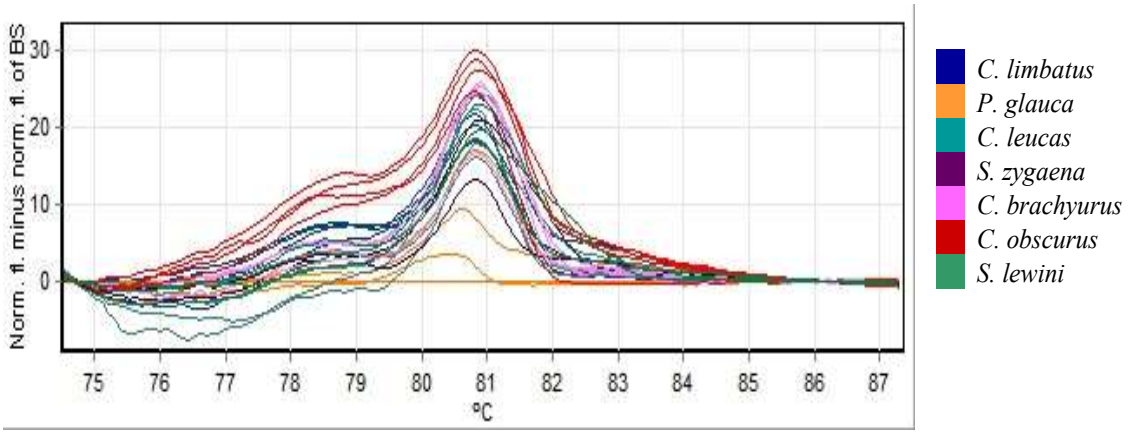

Figure S3.8: Difference plot for all additional shark replicate samples, using *Prionace glauca* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Prionace glauca* samples vs. temperature (°C).

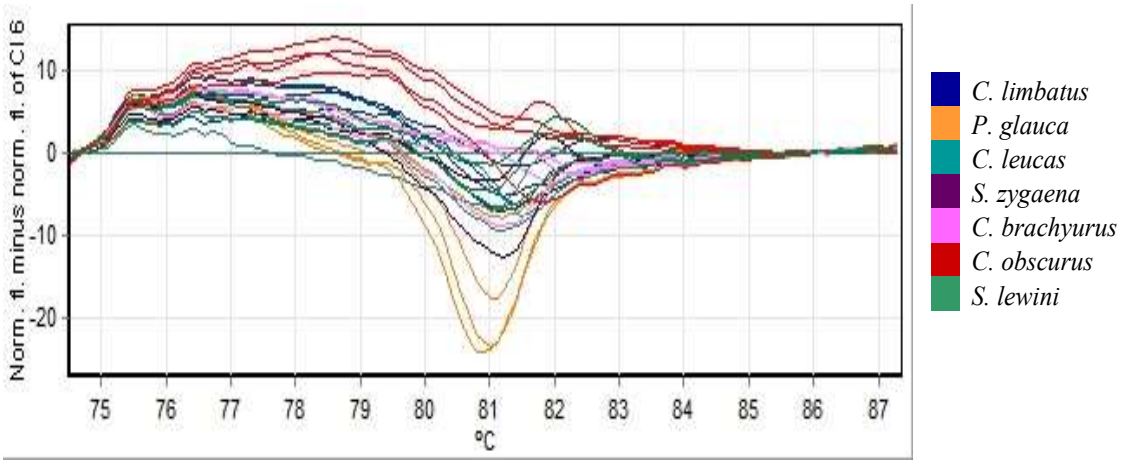

Figure S3.9: Difference plot for all additional shark replicate samples, using *Carcharhinus leucas* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Carcharhinus leucas* samples vs. temperature (°C).

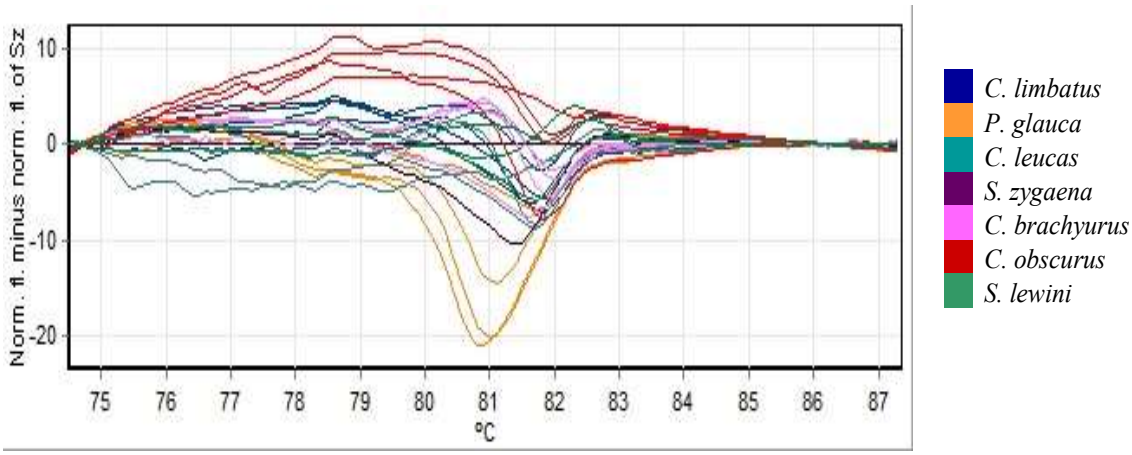

Figure S3.10: Difference plot for all additional shark replicate samples, using *Sphyrna zygaena* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Sphyrna zygaena* samples vs. temperature (°C).

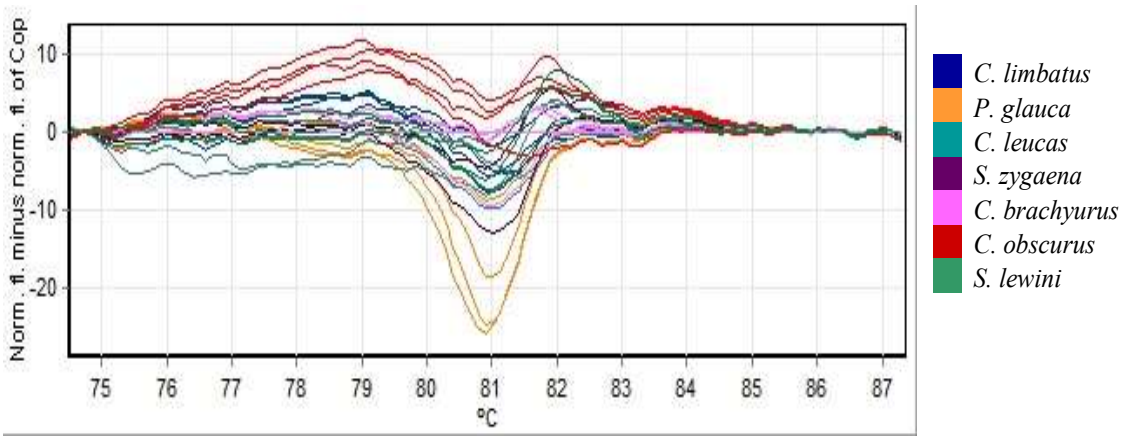

Figure S3.11: Difference plot for all additional shark replicate samples, using *Carcharhinus brachyurus* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Carcharhinus brachyurus* samples vs. temperature (°C).

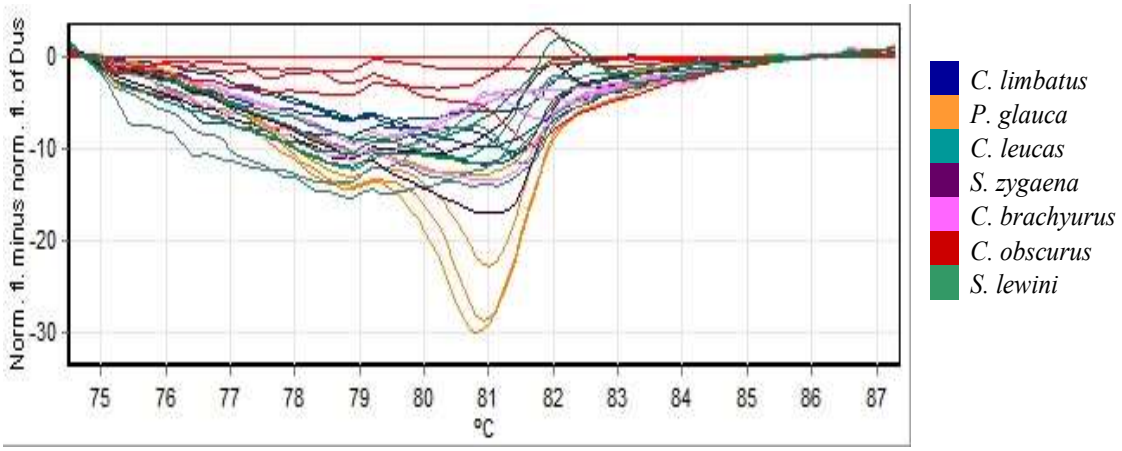

Figure S3.12: Difference plot for all additional shark replicate samples, using *Carcharhinus obscurus* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Carcharhinus obscurus* samples vs. temperature (°C).

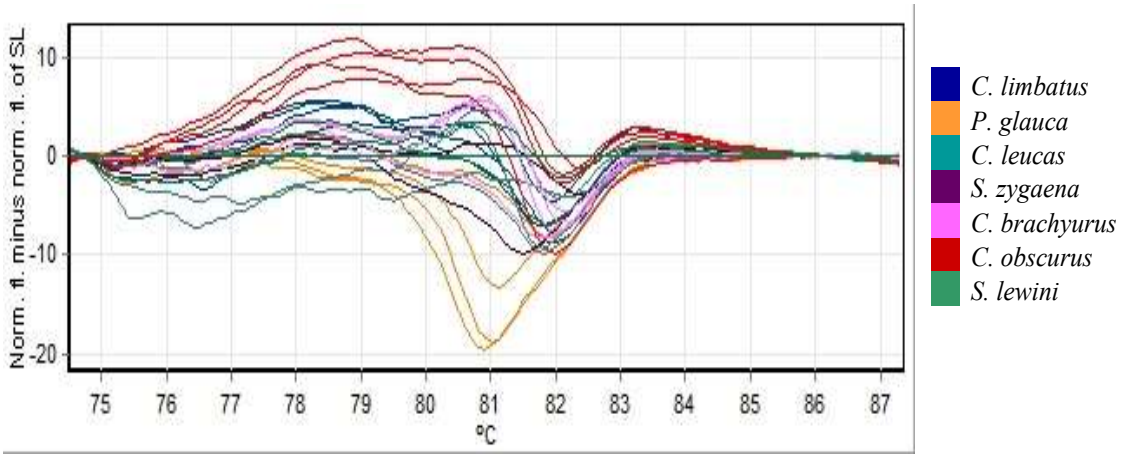

Figure S3.13: Difference plot for all additional shark replicate samples, using *Sphyrna lewini* as the baseline. Plotted as normalised fluorescence minus the normalised fluorescence of *Sphyrna lewini* samples vs. temperature (°C).